



CYTOLOGICAL CHANGES DURING
CHEMICAL CARCINOGENESIS IN MOUSE EPITHELIUM

The Appearance of
Cytochalasin B-Sensitive Microfilaments



HARRY L. MALECH

1972

YALE



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CYTOLOGICAL CHANGES DURING CHEMICAL CARCINOGENESIS
IN MOUSE EPITHELIUM

The Appearance of Cytochalasin B-Sensitive Microfilaments

Harry L. Malech

1972

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To Emily

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SUMMARY

The sequential changes in the fine structure of mouse epidermis treated with the chemical carcinogen 3, 4 benzopyrene were studied. Special attention was given to changes at the dermo-epidermal junction, changes in cell to cell contact, and the appearance and arrangement of those cellular organelles and structures usually associated with morphogenesis and cell movement. In addition cytochalasin B was used in vitro and in vivo as a chemical probe to investigate the nature of the 40 Å microfilaments which appear in the cells of the later developing carcinomas.

Observed changes in epidermal cells in carcinogenesis included increased numbers of ribosomes, a decrease in the number of tonofibrils, a decrease in the number of desmosomes connecting adjacent cells, appearance of large intercellular lacunae, formation of microvilli extending into these spaces, fragmentation of basement lamina, disappearance of basement lamina at sites of active tumor growth, and the appearance of basal cell pseudopods extending into the dermis. These changes are indicative of a loss of both differentiated cellular characteristics and normal intercellular relationships.

With increasing malignant change cells with a long cortical mesh of fine, 40 Å microfilaments, undifferentiated cytology, and pleomorphic shape with extensive pseudopodia

formation became increasingly abundant. When exposed to cytochalasin B in vitro or in vivo, the microfilaments became clumped and moderately disrupted. At the same time pseudopodia and microvilli of the pleomorphic cells are blunted. The structure of these filaments and their sensitivity to cytochalasin B places them in a class of microfilaments believed to have contractile properties and to be related to cell motility. Their presence may be correlated with the invasive properties of the malignant cells.

INTRODUCTION

Invasion and replacement of surrounding tissue is one of the primary behavioral characteristics of malignant neoplasms that distinguishes them from benign neoplasms and normal adult animal tissues. Various theories have been proposed to explain the origin of the motile force responsible for the peripheral spread of tumor cells into the adjacent normal tissue. One hypothesis is that increased pressure within the growing tumor provides the force for a passive intrusion of tumor cells into surrounding tissues (54,122,133). This, along with inflammation and perhaps release of proteolytic enzymes or cytotoxic substances by tumor cells (28,107), provides the necessary conditions for penetration of adjacent tissues.

On the other hand, the most widely accepted theory of tumor invasion suggests that active motile activity on the part of tumor cells is responsible for tumor invasion (2,13,34,65,90,123,124). Wolff, for example, has shown that a malignant tumor placed beside a normal tissue in culture results in invasion of the latter by tumor cells (123,124). This phenomenon is difficult to explain on the basis of buildup of pressure and passive movement of tumor cells. Furthermore, Wood studied in vivo movement of malignant carcinoma cells using a rabbit ear chamber and time-lapse cinematography. He found that under the same conditions, carcinoma cells and polymorphonuclear leukocytes

moved at similar rates while normal epidermal cells did not move at all. In addition the carcinoma cells continually shifted arrangements with respect to one another so that within the course of several minutes a random distribution might be replaced by an acinar, epidermoid, or squamous cell pattern (125).

Other processes such as release of cytotoxic substances, proteolytic enzymes or competition for nutrients certainly may play a role in breakdown of adjacent normal tissues, thus preparing the way for easy penetration, but the actual motile force for invasion is probably generated within the individual cells. Thus, explanations of the invasive behavior of malignant cells must take into account the mechanisms controlling their motile characteristics.

Since the earliest experiments in tissue culture it has been known that "amoeboid" type movement can be observed in vitro in a very wide range of metazoan cells (67). In 1962 Aberchrombie and Ambrose in an extensive review of the surface properties of cancer cells were able to cite abundant evidence indicating that the "amoeboid" type of movement seen in tissue culture with many normal and transformed cells might be similar to that involved in the invasive process of malignant cells in vivo. They preferred to call this movement "solid substrate locomotion" since it differed in some respects from the movement of amoebae (2).

Many of the important characteristics of motile cells have been defined in fibroblasts in culture (1,3,4,5,6,7,8,10, 26,50,52,61,116). The basic locomotive mechanisms, however, seem to be similar to other cells (105,115) and in epithelial

cells (53,64,66,117) which usually move as cohesive sheets. The fibroblast appears as a smoothly-contoured mound 2 to 4 μ in maximum thickness with a flattened prenuclear region called the leading lamella (5,60). A ruffling activity at the leading free edge of the cell occurs during movement (6,10,105,115). Adhesion between the cell and the substrate seems to occur most strongly just under the leading edge of the cells (5,115,117). The tip of the leading lamella bears one or more lamellipodia; transitory, mobile, sheet-like projections which appear in section for electron microscopy to look like microspikes (6,8).

Of special interest is a 500 to 1000 $\text{\AA}$ wide layer of 40 to 60 $\text{\AA}$ in diameter microfilaments lying longitudinally in the cell. The filaments are arranged as several long or short bundles of filaments almost parallel to each other just beneath the plasma membrane of the dorsal surface of the cell, and less frequently toward the very anterior end of the lamella or on the ventral surface (8,52).

Aberchrombie has suggested that a cell extends itself over the substrate by means of the assembly of new surface at its leading edge which is expressed in the projection of lamellipodia, which randomly form adhesions to the substratum. The body of the cell is drawn up to the new adhesions by the cortical system of contractile microfilaments noted above (8).

The appearance of cortical layers of microfilaments has been found by many workers to accompany such diverse cellular movements as cytokinesis (11,71,96,108,113), infolding of epithelial surfaces in morphogenesis (14,31,98,119,127), and the migration of epithelial cells in wound healing (64). All of

these activities require a contractile mechanism. Microfilaments are found in a location suggesting a contractile role for this structure. However, there is no evidence at present which definitely establishes the fact that microfilaments are contractile.

Cytochalasin B, a newly discovered fungal metabolic product, prevents cell locomotion (30,47,131,132), epithelial infolding (104,120,128), and cytokinesis (21,97,99). At the same time microfilaments are disrupted, decrease, or disappear from the cells while the morphology of other cytological structures is not affected. These observations implicate cortical layers of 30 to 70 Å microfilaments in cell motility. (Various diameters ranging from 30 to 70 Å have been reported for these microfilaments in the studies cited above.)

Because of the high degree of correlation between the presence of cortical bands of microfilaments and active cellular surface activity and movement, it is conceivable that the increased motility of cancer cells might be accompanied by the appearance of peripheral bundles of microfilaments at the fine structural level. To investigate the cytological changes in cancer cells, particularly filamentous structures related to motility, skin cancers were induced in mice by 3,4 benzopyrene and observed at the fine structural level. The effects of cytochalasin B on cytoplasmic filaments was tested.

Previous fine structure studies of the sequential changes in epithelium during chemical carcinogenesis were concerned mainly with changes in cytologic and nuclear organization, cell to cell contacts and surface alterations, and changes at the

dermo-epidermal junction (46,55,78,95,102,109,126). In particular, the appearance of pseudopodial extensions from basal cells and the dissolution of basement lamina has been interpreted by previous investigators as fine structural evidence of active invasion by transformed epithelial cells (55,95,109,126).

The present study will correlate the fine structural changes with recent knowledge concerning the function of cytochalasin B-sensitive microfilaments in cell movement. Special attention will be paid to those epidermal cells at the connective tissue interface since these are the cells that must actively invade and disrupt the surrounding tissues.

MATERIALS AND METHODS

Six week old female Balb/C mice were obtained from an inbred colony maintained at Yale University School of Medicine by Dr. William U. Gardner. 3,4 Benzopyrene and 1,2,3,4 dibenzanthracene obtained from Sigma Chemical Co. were used as carcinogen and non-carcinogenic control respectively (15,74). Both are polynuclear aromatic hydrocarbons of molecular weight 253.30 and 278.35 respectively. Both are soluble in acetone and both enter the epidermal cells of exposed mouse skin as shown by binding to cellular proteins (58,73).

Acetone solutions of 0.5% 3,4 benzopyrene or 0.5% 1,2,3,4 dibenzanthracene were prepared and stored in 1 ml aliquots in sealed glass vials. These were kept at 4° C in the dark for the duration of the experiment.

Previous dose-response studies have shown that an application of 100 µg. of 3,4 benzopyrene (0.5% in acetone) three times a week to mouse skin produces tumors within 10-15 weeks (83,129,130). The solutions were applied externally onto the skin drop-wise using a calibrated tuberculin syringe with a filed down 26 G needle (3 drops = 100 µg.). An identical regimen was followed using 1,2,3,4 dibenzanthracene.

The solutions were applied to the inner side of the pinna of the mouse's ear. The pinna proved to be especially advantageous as a site of application because it is easily observable

grossly, does not have to be shaved, and is very thin, making biopsies and fixation simple. Furthermore, the untreated epidermal surface lies within 0.5 mm of the treated surface allowing comparison in a single section. 3,4 Benzopyrene was applied to the right ear of twelve mice. A similar volume of pure acetone was applied to the left ear of these same mice as a control. 1,2,3,4 Dibenzoanthracene was applied to the right ear of six mice. Pure acetone was applied to the left ear of these mice. Application of the chemicals was continued for twenty-four weeks.

Tissue samples were taken from untreated mice and from treated mice at six weeks, twelve weeks and twenty-four weeks of exposure. Biopsies were taken of the part of the pinna containing the desired area of epidermis or tumor. Except in the case of deeply invasive carcinomas, this procedure did not require sacrifice of the mouse and allowed most of the mice to be followed for the full duration of the experiment. The observations and figures are representative of a survey of tissues from two mice at each stage of malignant transformation.

Cytochalasin B was obtained from Imperial Chemicals, Ltd. (Macclesfield, Cheshire, G. B.). For the in vitro experiments 10 mg of cytochalasin B was added to 0.4 cc of dimethyl sulfoxide and stored frozen. 0.02 ml of this solution was added to 5 ml of medium 199-1X (Unmodified, Earle's base) (Grand Island Biological Company, Grand Island, N. Y.). The final concentration was 100 µg cytochalasin B per ml of media. This concentration was chosen on the basis of previous dose-response studies. These studies demonstrated a significant decrease in

motility when a concentration of 10 ug/ml of cytochalasin B and 100 ug/ml were compared. However, even at 100 ug/ml the effects are reversible upon removal of cytochalasin B from the media (47). The final concentration of dimethyl sulfoxide was 0.4%. A control media was made by adding 0.02 ml of pure dimethyl sulfoxide to 5 ml of tissue culture media to make a concentration of 0.4%. After excision of a rapidly growing carcinoma, thin tissue slices were obtained with a razor blade. Several slices were placed in the cytochalasin B media or the control media for one hour at room temperature. At the end of this time the tissue slices were cut into blocks and fixed for electron microscopy.

For in vivo study, 0.1 cc of an 8 mg/ml solution of cytochalasin B in dimethyl sulphoxide was injected into the tail vein of a mouse with a rapidly growing carcinoma. One hour later the mouse was sacrificed and the tumor excised and fixed for electron microscopy.

All tissue samples were cut into small rectangular pieces to allow orientation and fixed for electron microscopy in the following manner. The tissue samples were placed in cold 3% gluteraldehyde in 0.05 M Na cacodylate buffer (pH 7.2), and stored for 2 hours at 4° C. They were then rinsed in cold buffer several times prior to fixation for 2 hours in 2% osmium tetroxide buffered with Na cacodylate (pH 7.2) at 4° C. After osmium tetroxide fixation the biopsy samples were washed first in Na cacodylate buffer (pH 7.2) and then several times in Michaelis buffer (pH 5.0). The tissues were then stained en bloc with aqueous uranyl acetate to enhance visualization of

membranes (25,42) by placing the tissues in 0.5% uranyl acetate in Michaelis buffer (pH 5.0) for 2 hours at room temperature. The tissues were then washed in Michaelis buffer (pH 5.0) and dehydrated in a graded series of ethyl alcohol and propylene oxide. After carefully orienting the rectangular tissue blocks to permit sectioning in a plane perpendicular to the surface of the epidermis, they were embedded in Maraglas (The Marblette Co., Div. of Allied Products Corp., Long Island City, N. Y.).

Thick sections were cut on a Porter-Blum MT-2 ultra-microtome with glass knives, stained on glass slides with 1% Toluidine blue, and surveyed for areas to be examined at the fine structural level. Thin sections cut with glass or diamond knives were stained with lead citrate (87) and examined with either an RCA EMU 3F or Hitachi 8B electron microscope.

OBSERVATIONS

NORMAL MOUSE EPIDERMIS

A. General Organization

The histology and cytology of normal interscapular mouse skin has been reviewed in previous studies at the light and fine structural level (49,109,102). Though the epidermis of the pinna of the mouse ear does not differ to any significant degree from that described previously, a review of the untreated pinna and its epidermis is necessary for a thorough understanding of subsequent changes in 3,4 benzopyrene treated tissue.

The entire thickness of a mouse ear is about one half to one mm. It is lined on both sides by an epidermis two to three cells thick and several layers of flat cornified squamous cells (Figs. 1,2). The dermo--epidermal junction is flat except where hair follicles extend into the deep layers of the dermis. Sebaceous glands are associated with these hair follicles. The cytoplasm of the cells in the sebaceous glands has a foamy appearance. In the deeper layers of the dermis are nerve bundles, adipose cells, fibroblasts, and muscle cells (Figs. 1,2). At the center of the pinna is a narrow, three to five cell layer of cartilage.

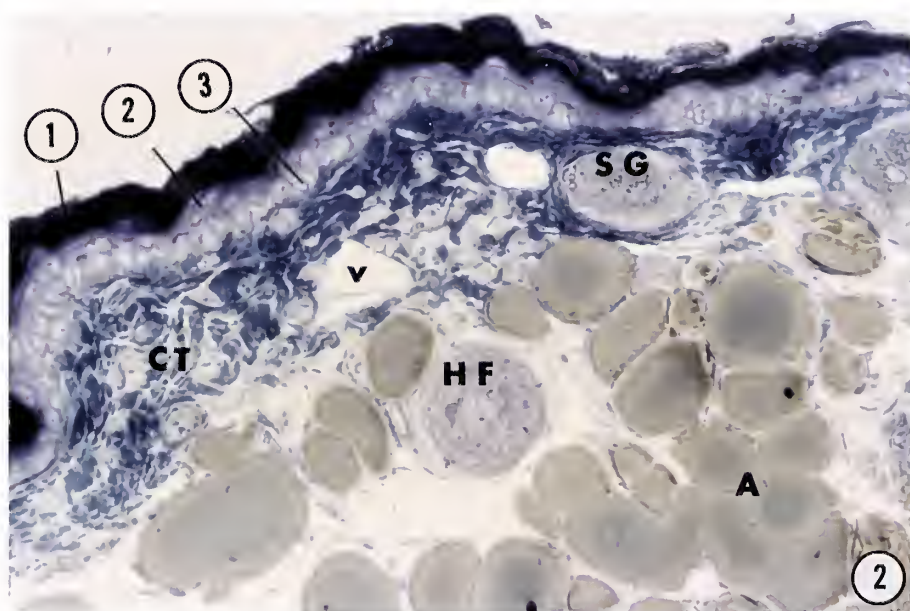
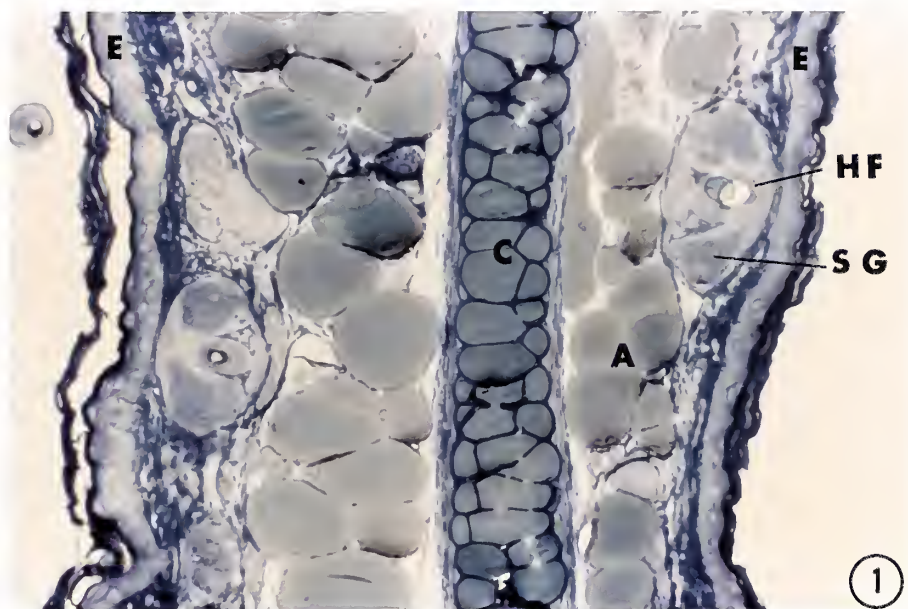
The keratinocytes in the lower layers of the epidermis are cuboidal or polygonal in shape. Overlying these cells are a

Figure 1. Cross section of normal mouse pinna. The pinna is lined on either side by a thin epidermis (E). Hair follicles (HF) with adjacent sebaceous glands (SG) lie immediately below the epidermis. Adipose cells (A) separate the epidermal structures from the central band of cartilage (C).

X 1140

Figure 2. Higher power of the inner surface of the mouse pinna near the base. Three layers can be distinguished in the epidermis, the stratum corneum (1), stratum granulosum (2), and stratum germinativum (3). Immediately beneath that is a moderately cellular layer of connective tissue (CT) containing fibroblasts and histiocytes. A layer of adipose cells (A) surrounded by a relatively acellular connective tissue occurs below the layer of fibroblasts. Hair follicles (HF) may extend partway into the layer of fat cells. Also seen beneath the epidermis are sebaceous glands (SG) and blood vessels (v).

X 1800



few flattened scales of the stratum corneum, some of which are in the process of desquamation (Fig. 3).

B. Cytologic Structures

Two types of keratinocytes can be distinguished: a dark and a light cell (Fig. 4). Light cells are scattered individually or in small groups among the more numerous dark cells. The light cells are characterized by a lower electron density and fewer organelles than dark cells. In addition, a few melanocytes are found in the epidermis (Fig. 3).

1. Nucleus and Organelles

The nuclei of the keratinocytes are centrally placed and round. The nuclei contain dark clumps of heterochromatin, which tend to lie towards the periphery. A nucleolus is occasionally seen in the nucleus. Sometimes the nuclear contour is indented. The cells contain one or two Golgi complexes usually located in the nuclear indentation or adjacent to the nucleus (Figs. 4,5). The Golgi apparatus consists of several parallel layers of flattened, elongated membrane-bound cisternae. Surrounding this may be several clear vacuoles or vesicles. The Golgi complex is not a prominent feature; in most cells it occupies only a small part of the cytoplasm. Other than the Golgi and its associated vesicles, the cytoplasm of the keratinocytes contains very little membranous material. A few short cisternae of rough-surfaced endoplasmic reticulum occur (Fig. 5). There is a moderate number of ribosomes in these cells. These are scattered throughout the cytoplasm, and tend to occur singly and are not ordinarily aggregated into polysomal clusters. Mitochondria are present in

Figure 3. Full thickness of normal epidermis from pinna of mouse ear. The dermis (D) contains orthogonally arranged collagen fibrils (C). A basement lamina (arrow) underlies the epithelium. The basal layer of the epidermis is composed of a row of cuboidal epithelial cells or keratinocytes. An occasional melanocyte (Mel) is interspersed in this layer. The keratinocytes contain bundles of tonofilaments. Above this layer the cells are more elongated and in the stratum corneum (upper right) are flattened scales lacking nuclei.

X 18,700

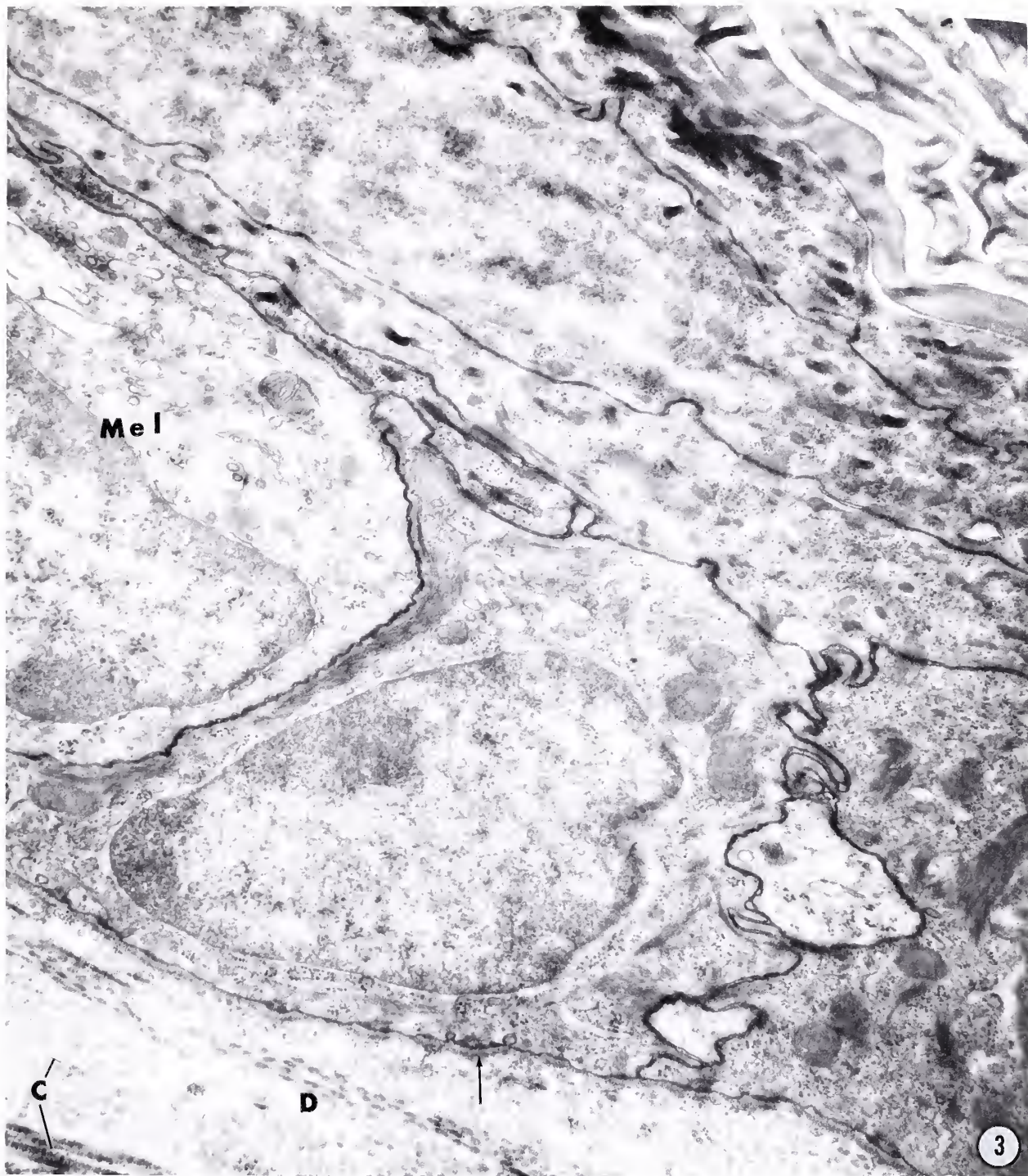


Figure 4. Higher magnification of normal epithelial cells. Two cell types can be distinguished: a light cell of low electron density (upper left) and dark cells with more cytoplasmic organelles. The cells contain a Golgi apparatus (G), tonofibrils (T), and free ribosomes (R). A nucleus is seen in the upper right.

X 56,700

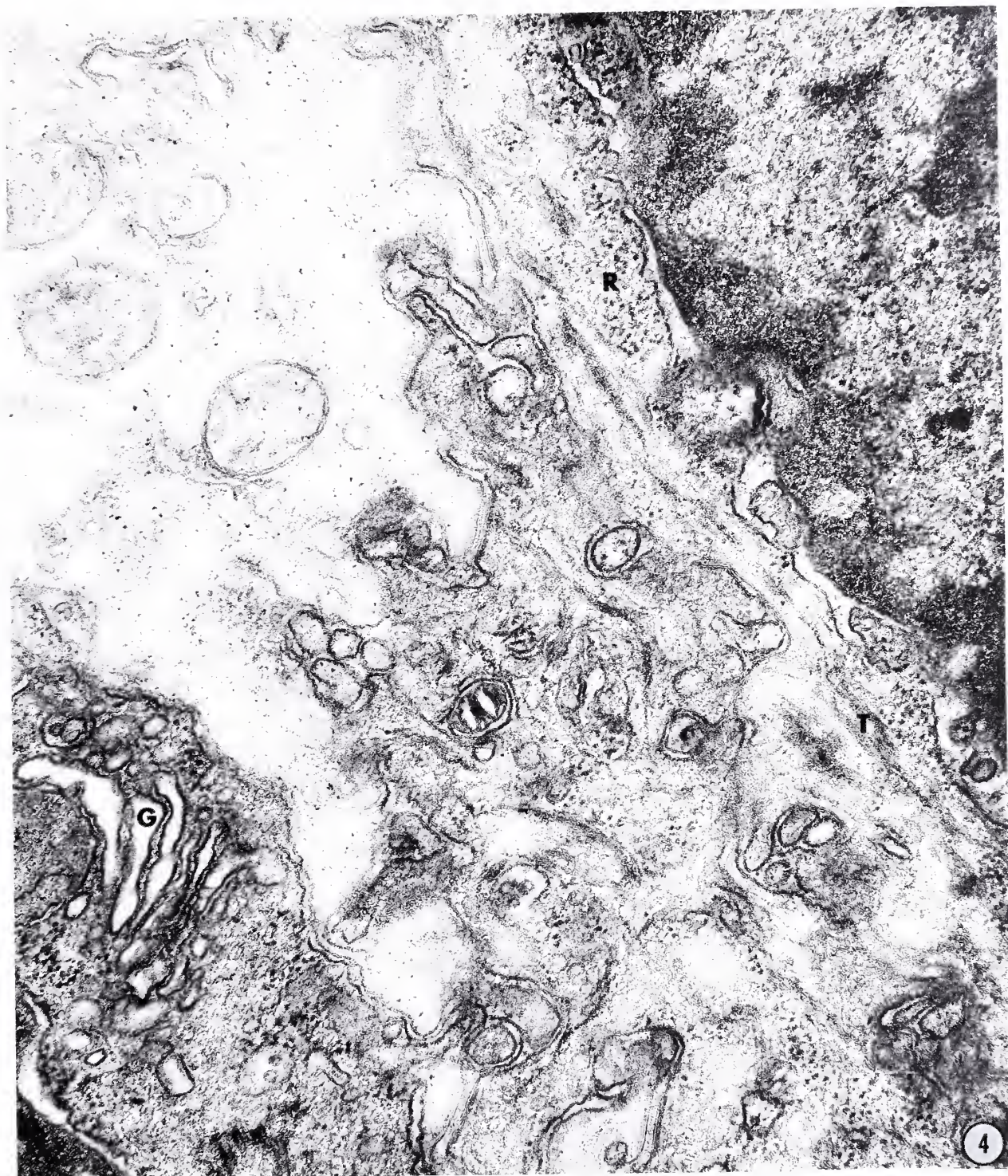
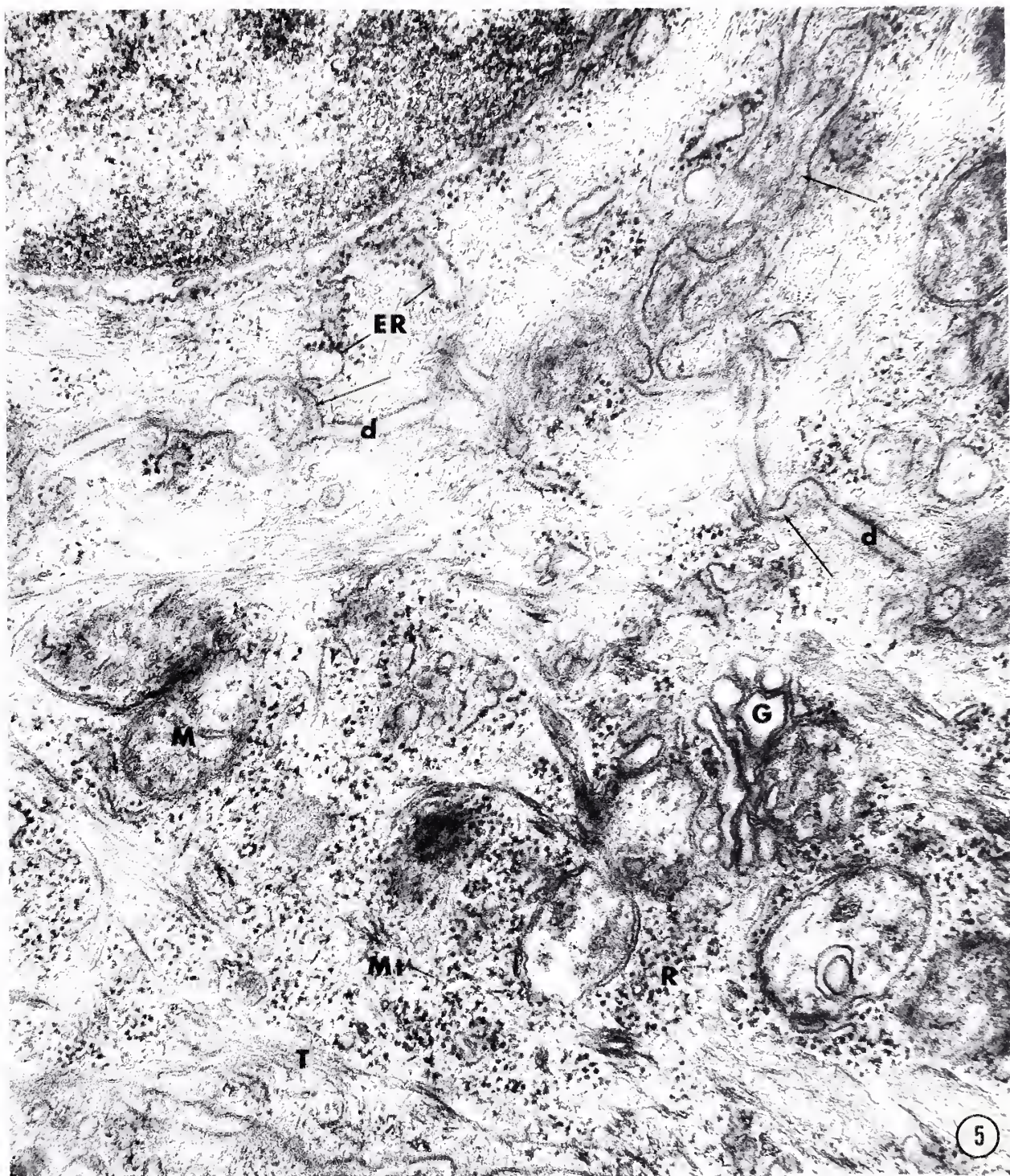


Figure 5. Normal epithelial cells. Three cells are seen separated by a relatively narrow intercellular space (arrows). Several desmosomes (d) can be seen between adjacent cells. The cells contain a Golgi (G), tonofibrils (T), free ribosomes (R), as well as a few short cisternae of rough-surfaced endoplasmic reticulum (ER). Mitochondria (M) and an occasional microtubule (Mt) occur. A part of a nucleus can be seen in the upper left.

X 57,200



small to moderate numbers. They are round or elliptical in shape with some variation in size. Extending only partway across the mitochondria, the fingerlike cristae are embedded in an amorphous moderately electron dense matrix (Fig. 6).

2. Filaments

The most prominent cytoplasmic feature of keratinocytes are bundles of tonofibrils weaving through the cytoplasm. These bundles may be short or may extend over the entire width of the cell. In basal cells they are oriented predominantly perpendicular to the base and appear to insert into desmosomes at the sides and top of cells and into the hemidesmosomes along the base (Figs. 1,6). In cells in the upper layers, tonofibrils are oriented more parallel to the surface of the epidermis. Other organelles are excluded from the area of the cytoplasm occupied by these bundles (Fig. 5).

The tonofibrils are composed of tonofilaments approximately $100 \text{ \AA}$ in diameter (101). An electron-dense material is associated with the tonofibrils in cells of the upper layers (Fig. 7). Some tonofilaments are not organized into bundles, but lie singly in the cytoplasm. The filaments are often wavy or curved and usually cannot be followed for more than a half a micron.

A few microtubules are seen in the more differentiated epidermal cells of the stratum germinativum (Fig. 5). The microtubules when seen are oriented parallel to the elongated axis of the flattened cells of the stratum corneum. The microtubules have the typical appearance of a straight hollow tube $240 \text{ \AA}$ wide with $50 \text{ \AA}$ walls and up to several hundred μm in

length (84). No other types of fibers or filamentous processes are seen in normal untreated epidermal cells.

3. Intercellular Relationships

The epidermal cells are closely packed and an intercellular space cannot be discerned at the light microscopic level. At the fine structural level, there is some interdigitation of cells with processes of one cell indenting the contour of the adjacent cell (Fig. 3). Adjacent cell membranes are aligned parallel to one another separated by a relatively uniform intercellular space of 150 to 300 Å. The intercellular space is filled with a moderately electron-dense amorphous material (Fig. 5).

The most frequent type of intercellular attachment is the desmosome. These can be vaguely discerned at the light microscopic level as dense spots. At the fine structural level they have the typical structure of filaments inserting into dense material on the inner aspect of the plasma membrane, and a widened intercellular space containing a plaque of dense material (42) (Fig. 7). The desmosomes are 0.2 to 0.5 μ in length and occur along the interdigitating processes and also where the cell borders are straight.

Half or hemidesmosomes cover almost a third of the basal plasma membrane adjacent to the dermis (Fig. 6). They are composed of a thick plaque on the inner side of the membrane into which the tonofibrils insert, and a thin extracellular plaque just below the membrane. Between the hemidesmosomes along the basal plasma membrane, pinocytotic invaginations and vesicles are seen. These usually contain some electron-dense material

adherent to the membrane (Fig. 6).

A close (gap) junction or tight junction (42,86) can occur between cells. In the gap junction, the membranes approach very closely but a small intercellular space of $20 \text{ \AA}$ remains between adjacent outer membrane leaflets. In tight junctions, the outer leaflets of the unit membrane appear fused. Between the basal cells of the epidermis a focal gap junction appears to seal off the epidermal intercellular space immediately above the base (Fig. 6). The next specialized junction to occur farther up the intercellular space is a desmosome, which may be almost adjacent to the gap junction or up to one micron away. Occasional tight junctions, as well as gap junctions, occur between basal cells and cells of the overlying stratum. The tight junctions may be focal or up to $500 \text{ \AA}$ in length (Fig. 7). The differences in length are probably due to the plane of sectioning.

4. Basement Lamina

A basement lamina underlies the epidermis and is separated from the basal plasma membrane by a clear space of $200 \text{ \AA}$. The basement lamina is a continuous, uniform $200 \text{ \AA}$ wide band of moderately dense, finely filamentous material which closely parallels the base of the epidermis (81,93) (Fig. 6). Beneath hemidesmosomes the basement lamina often appears more dense, and occasionally fine anchoring fibers from the dermis appear to insert at these sites.

C. Dermis

In the dermis, collagen fibrils lie parallel to the base of the epidermis. These possess the periodic structure usually

Figure 6. Base of normal epidermis. The dermis (D) contains orthogonally arranged collagen fibrils (C) of uniform diameter. A basement lamina (BL) underlies the epithelial cell. Hemidesmosomes (hd) occur at the base of the epithelial cell. Pinocytic vesicles (p) pinch off from the basal plasma membrane between hemidesmosomes. Between epithelial cells at the base, a gap junction (g) seals off the intercellular space. Further up the intercellular space a desmosome (d) can occur. The cell contains mitochondria (M), free single ribosomes, and tonofibrils (T) which insert into the desmosomes. A nucleus is seen in the upper left.

X 45,000

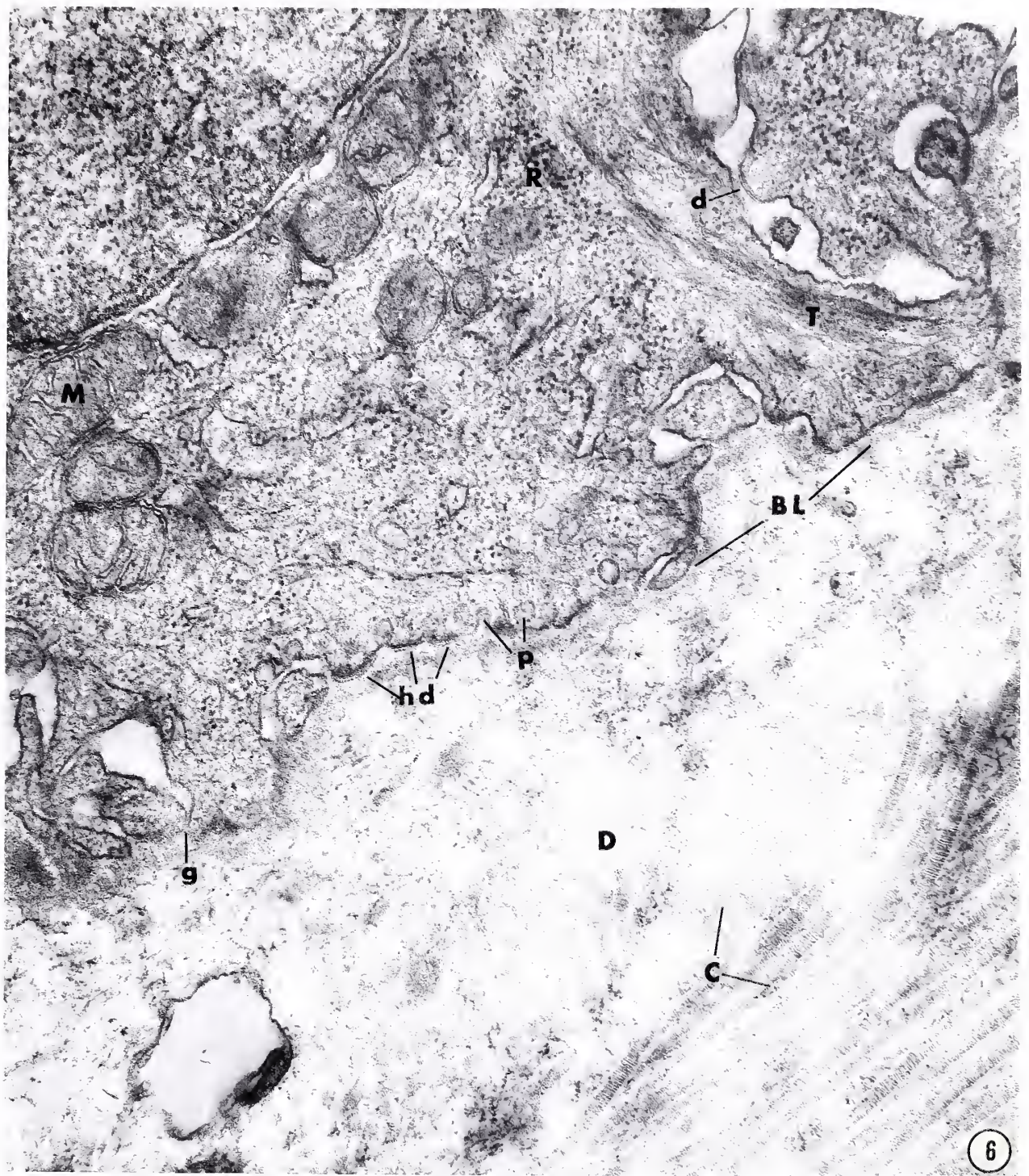


Figure 7. High magnification of a desmosome (d) in the upper layers of the epidermis. Tonofibrils (T) can be seen to insert into the desmosome. The desmosome has a typical 9 layered appearance. Near the desmosome the intercellular space at first widens, and then is obliterated at a short tight junction (t).

X 102,000

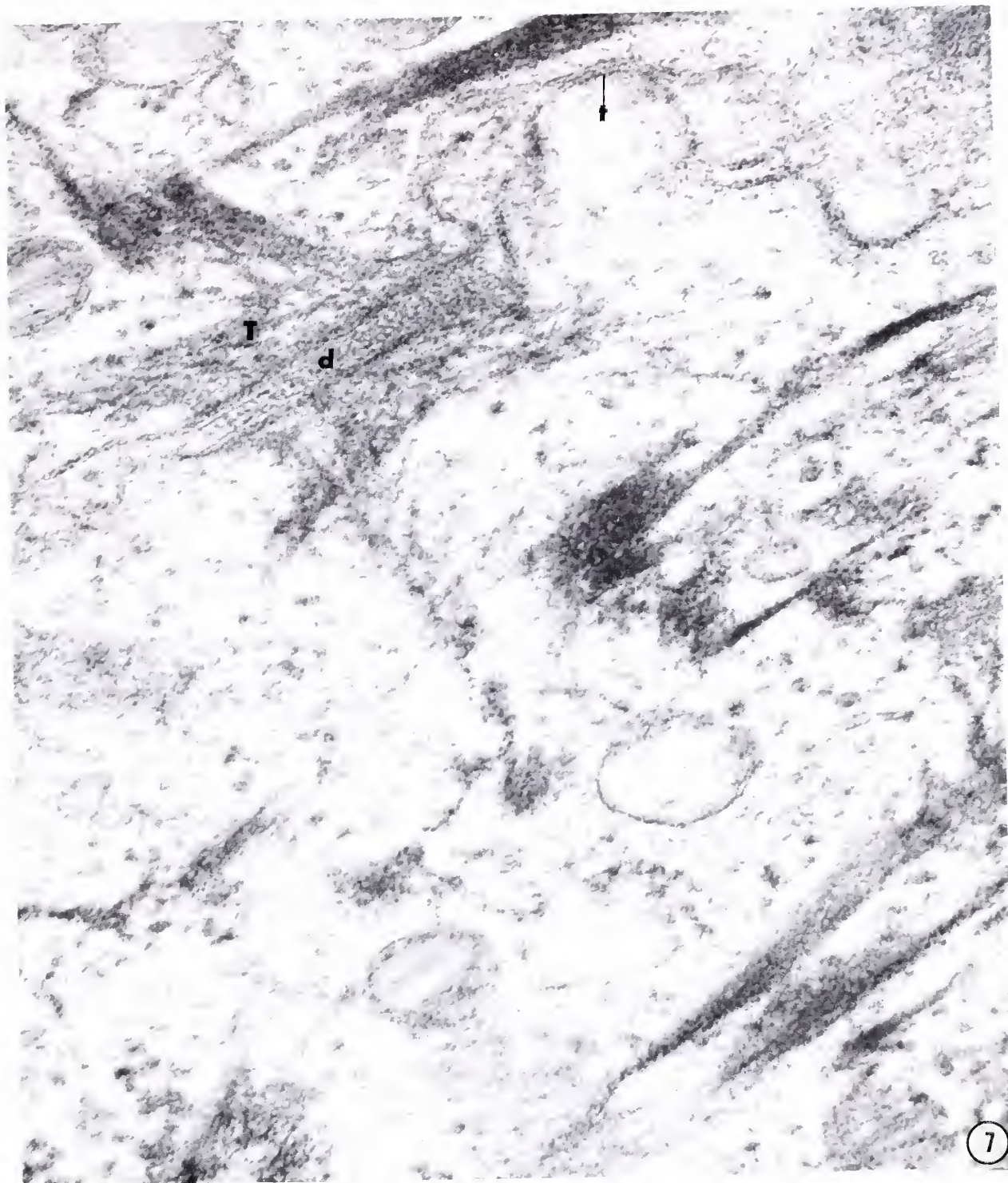
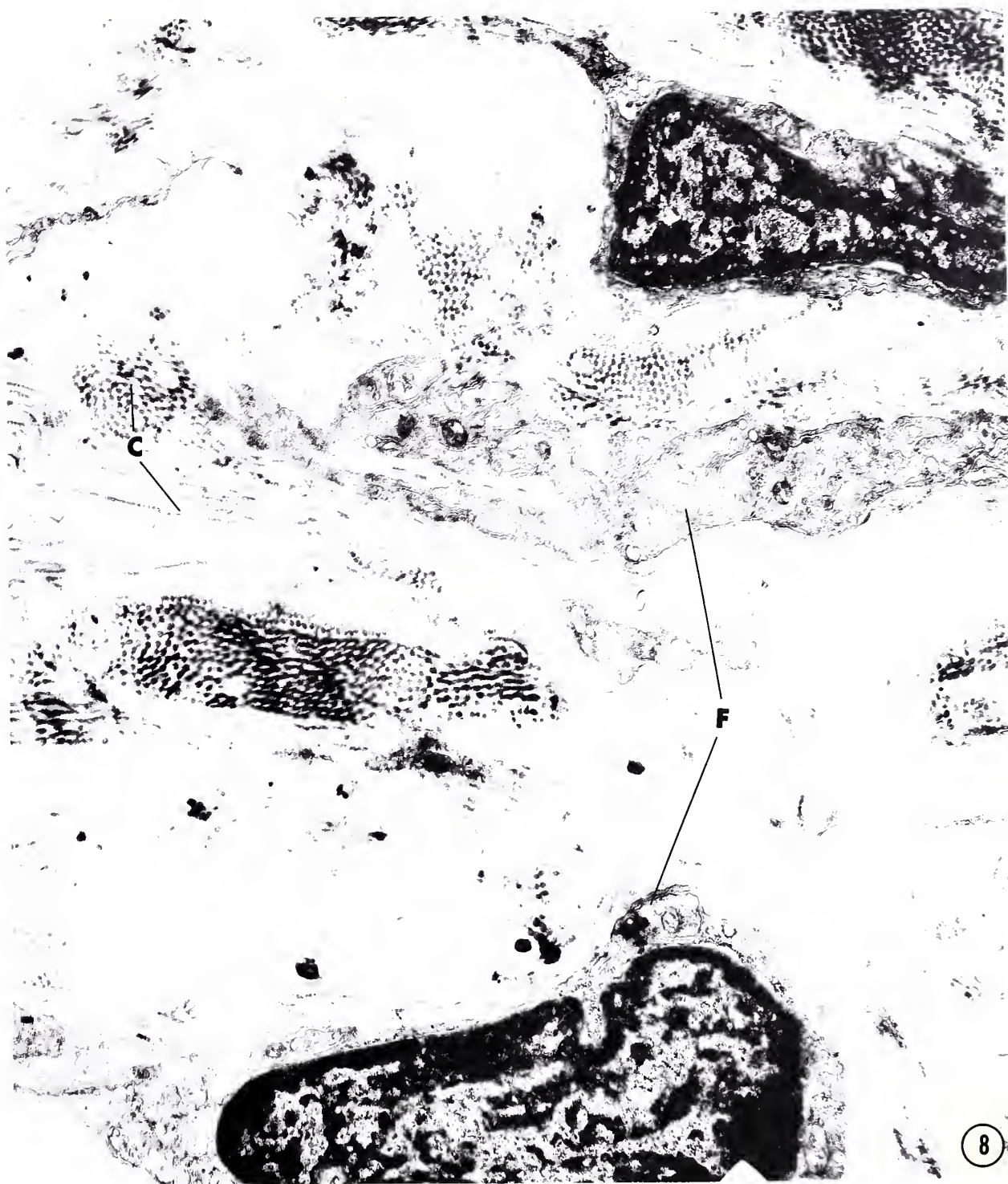


Figure 8. Normal Dermis. Fibroblasts (F) can be seen within the matrix formed by orthogonally arranged bundles of collagen fibrils (C).

X 16,800



associated with this fibril type. The collagen fibrils are associated into large bundles. Though all the bundles are parallel to the base of the epidermis, some bundles are arranged at right angles (Fig. 6). Occasionally part of a fibroblast is seen, always separated from the epidermis by several layers of collagen bundles. The fibroblasts are easily distinguished from the epidermal keratinocytes because of the lack of tonofibrils or desmosomes and the presence of moderate amounts of rough endoplasmic reticulum. In addition, there is no basement lamina separating the fibroblasts from the surrounding connective tissue (Fig. 8).

6 TO 8 WEEKS 3,4 BENZOPYRENE EXPOSURE

A. General Organization

1. General Epidermis

On the treated surface of the pinna, the epidermis has increased to six or eight cells in thickness with a thick layer of flat cornified scales. The epidermis on the untreated side of the same pinna is also affected but not to the same degree; the thickness varying from normal to five or six cells. The hair follicles are hyperplastic (Fig. 9). Sebaceous glands have disappeared or become small and atrophic (Fig. 9). The cells no longer have a foamy appearance, but more closely resemble keratinizing epithelial cells. The dermis is very cellular (Fig. 9). Increased numbers of fibroblasts and inflammatory cells now occupy areas where extracellular fibrils were formerly the main component.

In the untreated surface and most of the treated surface,

the hyperplastic change is not accompanied by any discernible difference in cell shape, packing, or nucleation. At the fine structural level, there are a few small focal dilations of the intercellular spaces. Desmosomes and hemidesmosomes are numerous; a gap junction seals off the intercellular space at the base of the epidermis; tonofibrils are common with the usual orientation; ribosomes are moderately abundant and occur singly; smooth and rough endoplasmic reticulum is infrequent; the Golgi is not increased in size; and the basement lamina is continuous, of uniform thickness, and parallel to the base of the epidermal cells. Thus, the majority of the epithelial cells are normal in cytological appearance.

2. Focal Areas of Excessive Hyperplasia

Within the treated epidermis, but not in the opposite surface, are scattered areas of more marked hyperplasia (Fig. 9). These areas differ markedly in fine structure from the rest of the epidermis. The epidermis in these areas may be twelve or more cells thick. This focal increase in cells bulges into the dermis as well as causing a microscopic bump on the surface of the epidermis. The epidermal cells are somewhat pleomorphic in shape with many small pseudopodia or microvillous processes extending into the intercellular spaces (Fig. 10). Distinction between light and dark cells is no longer as evident.

B. Cytological Structure

1. Nucleus and Organelles

The nuclei of the keratinocytes are round or elongated in shape. They contain less condensed heterochromatin, but nucleoli

are more prominent (Fig. 10). In the cytoplasm, Golgi complexes are more abundant and have a greater number of small vesicles and vacuoles associated with them (Fig. 11). Vesicles and small vacuoles are common throughout the cytoplasm, and frequently occur near the cell surface. Cisternae of rough-surfaced endoplasmic reticulum are also more common. These are dilated and contain flocculent material. Free ribosomes fill much of the cytoplasm not occupied by other organelles. The ribosomes of many cells show a greater tendency to be grouped in small clusters. In other cells a larger proportion of ribosomes occur singly (Fig. 11). A number of lysosomes and multivesicular bodies may occur in the cytoplasm.

There appears to be a slight increase in the number of mitochondria. Most of the mitochondria are round or elliptical and some are large and pleomorphic. The matrix is less dense than in the untreated cells. Some of the mitochondria have an electron-dense round body in the matrix (Fig. 11).

2. Filaments

The cells maintain their extensive population of tonofibrils (Fig. 11). There is some disorganization of the usual orderly alignment of fibrils within the cell. However, the predominant orientation in basal cells is still perpendicular to the basal lamina. In the upper cells the orientation is more parallel to the surface of the epidermis. Some of the fibrils are long and extend into cellular processes to insert into a desmosome. Other fibrils are short and these frequently have dense amorphous material associated with them.

Microtubules in untreated tissue are seen only in small

Figure 9. Six to eight weeks exposure to 3,4 benzopyrene, focal area of excessive hyperplasia. The epidermis (E) here is twelve cells thick. Elsewhere the hyperplasia may not be as great, amounting to only six to eight cells in thickness. Of particular interest are the widened intercellular spaces (arrows), seen even in this light micrograph. The hair follicles (HF) have also undergone hyperplasia and the sebaceous glands are no longer evident. Beneath the epidermis the dermis (D) is quite cellular, containing inflammatory cells as well as fibroblasts and other connective tissue cells.

X 1800

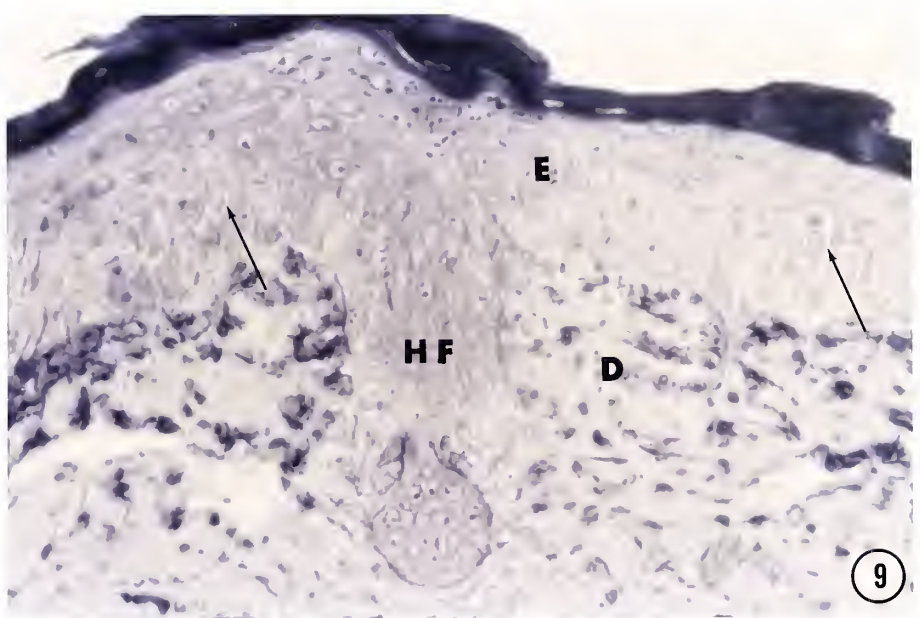


Figure 10. Six to eight weeks of exposure to 3,4 benzopyrene; epidermis of mouse pinna; focal area of excessive hyperplasia. The dermal collagen fibrils (C) are more variably oriented. Within this matrix an occasional fibroblast (F) is seen. The base of the epidermis is irregular, but the basement lamina (BL) is continuous and follows the contour of the base approximately. The epithelial cells are quite irregular in outline with numerous microvilli (Mv) projecting into the intercellular spaces (ICS). The latter have widened into large lacunae. Some amorphous clumps of material (arrows) occur in the intercellular space adherent to the cells. Desmosomes (d) are seen at the tips of some villous processes. The cells contain several Golgi complexes and a large amount of endoplasmic reticulum. The nuclei contain only a small amount of heterochromatin (h). An eccentrically placed nucleolus (n) occupies one of the nuclei.

X 10,600

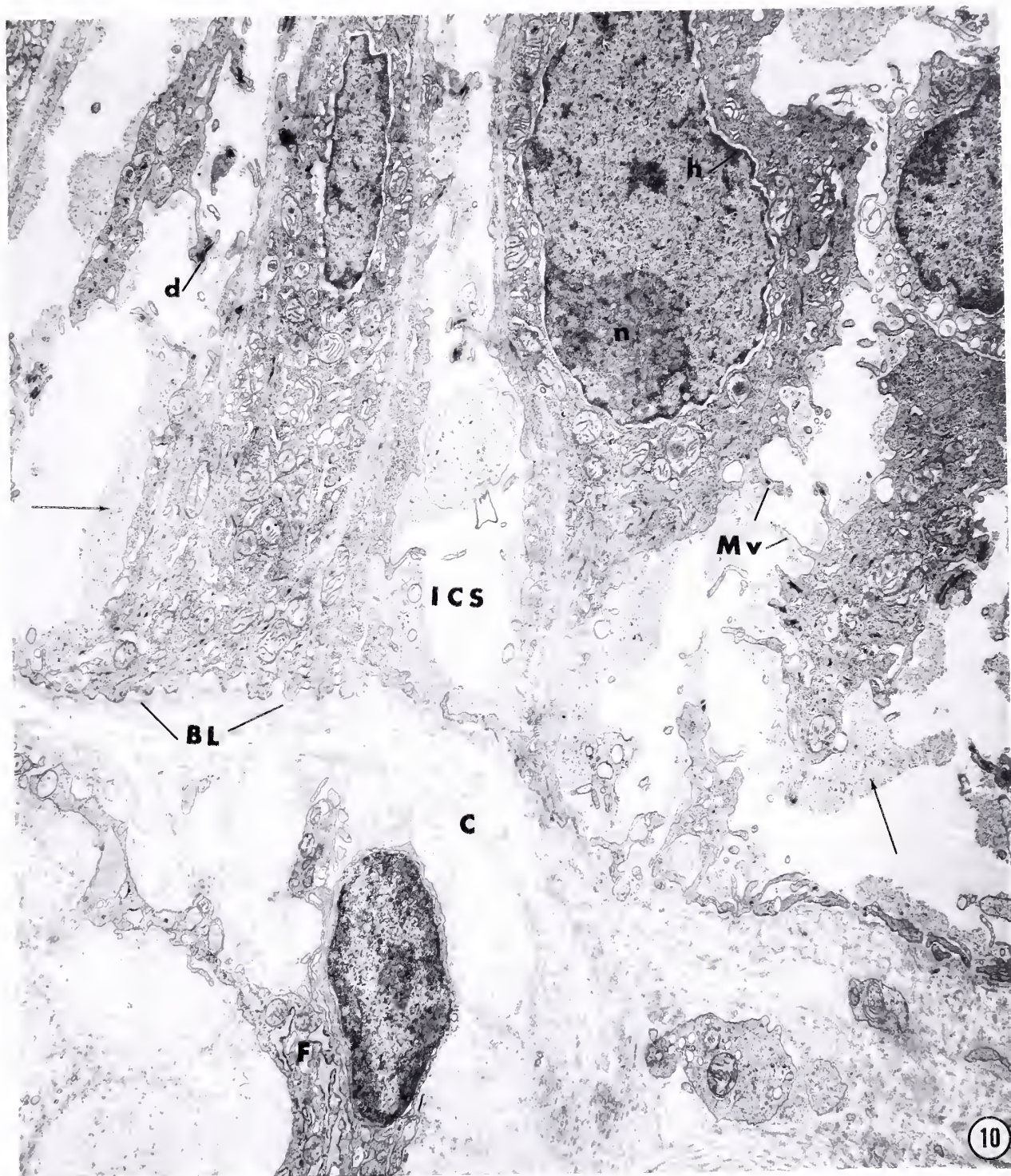
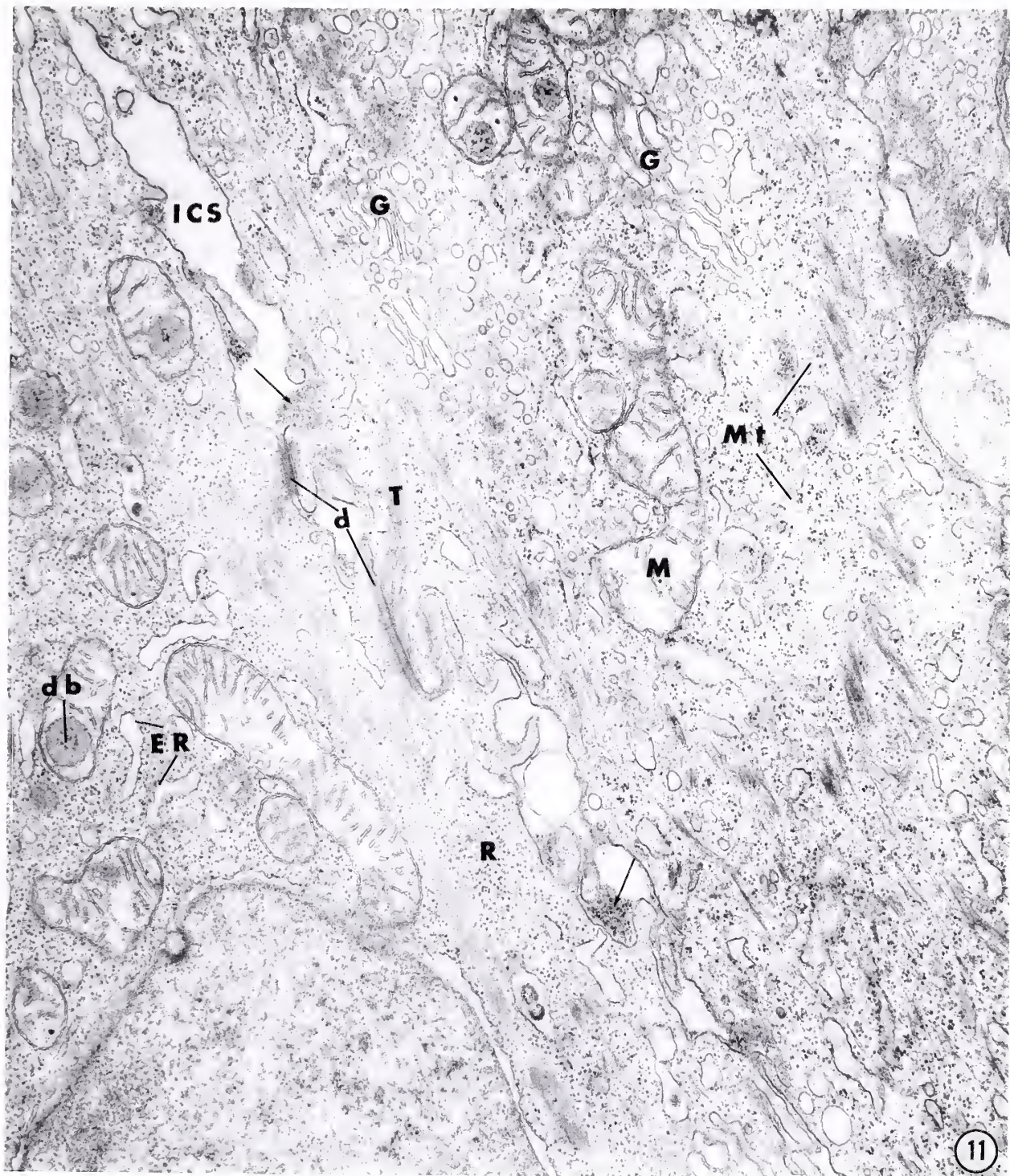


Figure 11. Higher magnification, six to eight weeks benzopyrene, focal area of excessive hyperplasia, two cell layers above the base. The intercellular space (ICS) is wider than normal. Amorphous material (arrows) can be seen adherent to the cell surface. Desmosomes (d) occur and tonofibrils (T) are abundant inserting into the desmosomes. Golgi complexes (G) are increased in number and size. Many vesicles are associated with them. Free ribosomes (R) occupy a larger part of the cytoplasm. In the cell on the right there is an increased tendency for the ribosomes to be associated as free polysomes and membrane-bound polysomes. There is an increased amount of rough-surfaced endoplasmic reticulum (ER). Many microtubules (Mt) course through the cytoplasm oriented in the direction of the tonofibrils. The mitochondria (M) are more numerous, large and irregular in shape, with electron-lucent matrix. Many contain a round dense body (db) in the matrix.

X 29,100



numbers in the cells of the upper layers. In these focal areas of excessive hyperplasia in the six week treated skin, many microtubules are seen in the cells of all layers. They may be followed for several hundred millimicrons, and are oriented perpendicular or parallel to the local arrangement of tonofibrils (Fig. 11).

In addition to tonofibrils and microtubules, a new population of filamentous structure appears at this time in a very small number of cells. These structures are microfilaments 40 Å in diameter, organized into loose bundles or networks. They tend to be more numerous in the villous processes (Fig. 12). In the latter the filaments appear to extend the length of the process. At high magnification each individual filament is seen to be quite short, extending only a few hundred Å. Occasionally small clumps of microfilaments are also seen in focal areas just under the plasma membrane at the periphery of some cells. Other organelles are excluded from the areas occupied by the microfilaments (Fig. 12).

3. Intercellular Relationships

The cells appear at the light microscopic level to be slightly separated from one another by clear spaces (Fig. 9). At the fine structural level these spaces are seen to be large intercellular lacunae, some of which are several microns across (Fig. 10). Masses of amorphous extracellular material adhere to the cells. Occasionally an inflammatory cell is seen in the intercellular lacunae (Fig. 12). Villous and cytoplasmic extensions project into these intercellular spaces.

Desmosomes are rare between basal cells and occur where

Figure 12. Six to eight weeks exposure to benzopyrene.

Rarely within the hyperplastic epidermis an epidermal cell like that seen in the upper part of the photograph occurs. This epidermal cell contains 40 $\text{\AA}$ microfilaments (Mf), dilated Golgi apparatus (G), spike-like clumps of tonofibrils (T), and many single ribosomes (R). The intercellular space is quite dilated; and a part of an inflammatory cell (lower left) can be seen within this space.

X 42,200



ends of long cellular extensions meet in the lacunae (Fig. 10). There are many desmosomal attachments between the cells in the upper layers. Often these upper cells appear separated, remaining attached only by the desmosomes at the tips of stout projections from adjacent cells (Fig. 13). The desmosomes are not altered in structure (Fig. 14).

At the base of the epidermis hemidesmosomes are unaltered in structure or frequency. The base of the epidermal cell is quite irregular with many small blunt projections into the dermis (Fig. 15). The hemidesmosomes are located on these projections. Pinocytotic vesicles are not as commonly seen at the base as in normal untreated epidermis.

Gap junctions and tight junctions are still seen occasionally between extensions of cells in the upper layers. However, at the base of the epidermis a gap junction no longer seals off the intercellular space. Basal projections of adjacent cells may be separated by an 0.2 to 0.5 μ space (Fig. 15).

4. Basement Lamina

Below the basal cells the basement lamina approximately follows the irregular contours of the basal cell plasma membrane (Fig. 15). At hemidesmosomes the distance between the basement lamina and plasma membrane is about 200 to 300 Å, but elsewhere the basement lamina does not follow the plasma membrane as closely.

C. Dermis

Beneath the basement lamina in the dermis, the usually orderly arrangement of collagen fibers is disturbed (Fig. 15).

Figure 13. Six to eight weeks exposure to benzopyrene. In the upper layers of the hyperplastic epidermis, desmosomes (d) are still common between epidermal cells. They occur at the ends of blunt cellular projections. The cells almost seem to be stretched apart, with intercellular contact occurring only at the desmosomes. Tonofibrils (T) are abundant and insert into the dense plaque on the intracellular side of the desmosome.

X 56,700

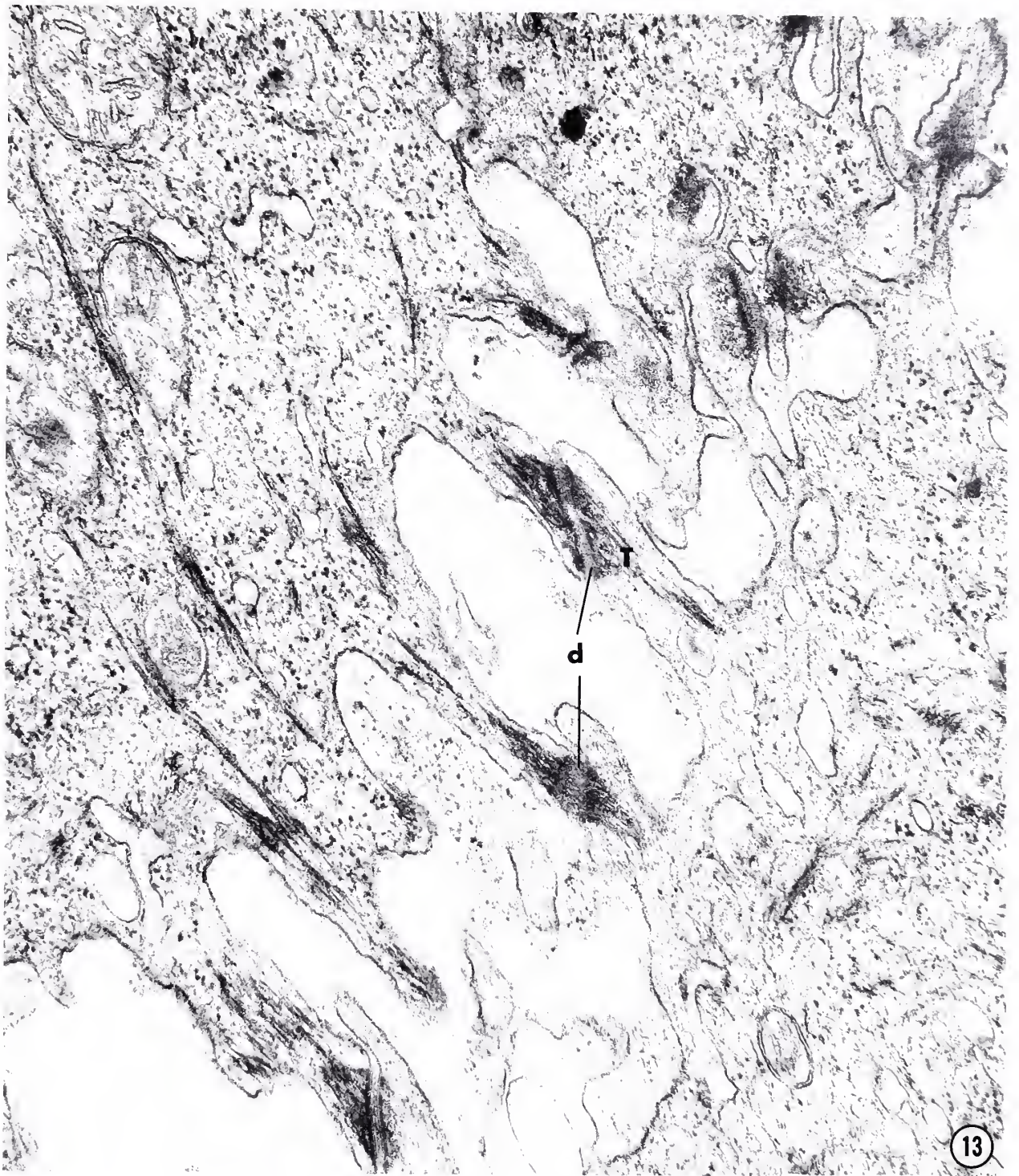


Figure 14. Six to eight weeks exposure to benzopyrene. Very high magnification of two desmosomes (d) at the ends of blunt cellular extensions. The desmosomes have the typical nine layered appearance with tonofibrils (T) inserting into the dense plaque on the intracellular sides of the desmosome. In the upper left hand corner is a tonofibril in which the individual tonofilaments can be distinguished. In addition a small segment of a microtubule (Mt) can be seen.

X 144,000

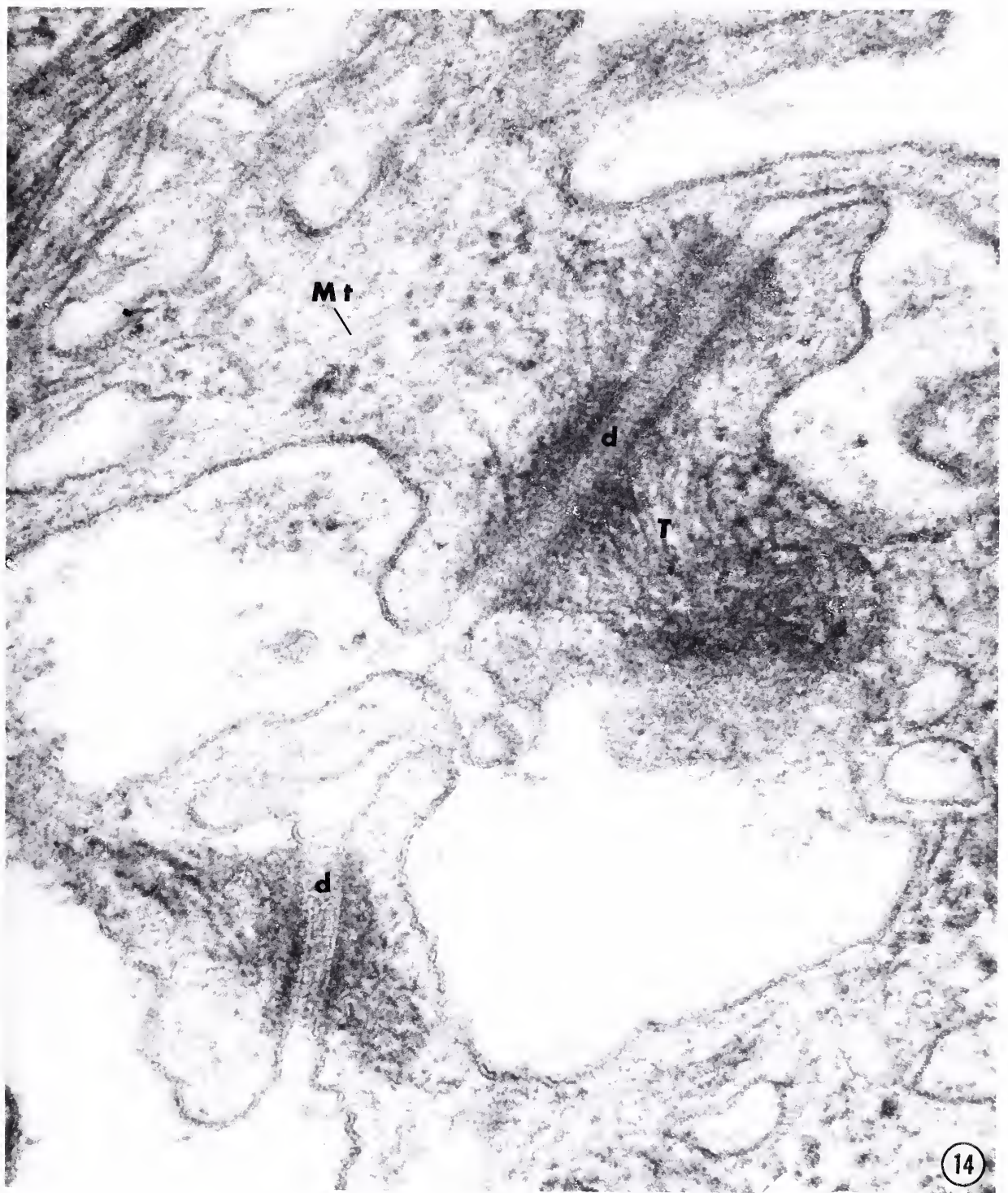
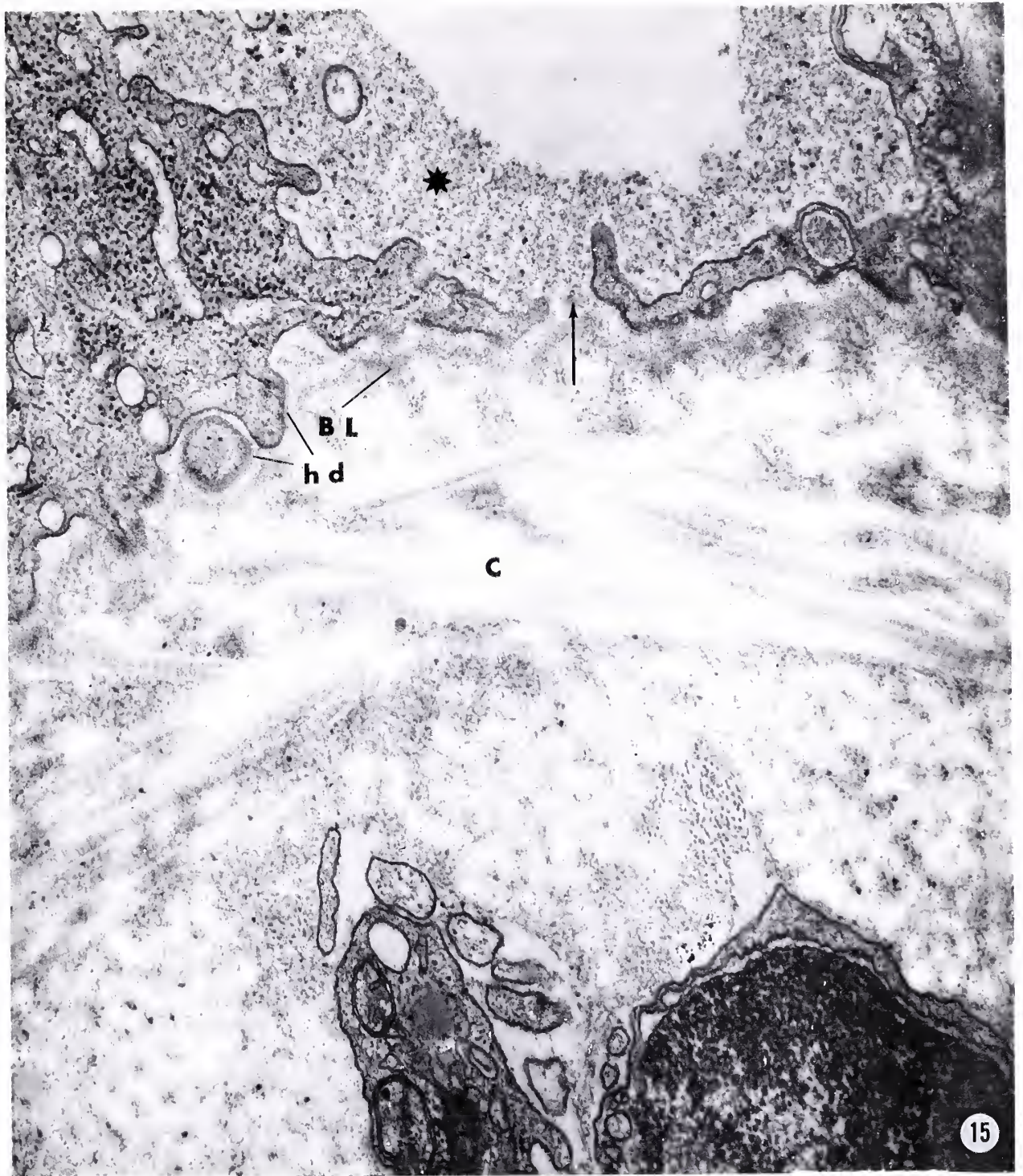


Figure 15. Six to eight weeks exposure to benzopyrene; base of the epidermis. The collagen fibrils (C) in the dermis are more irregularly oriented and of variable diameter. The basement lamina (BL) underlies the epithelial cells and is unbroken, but irregular in outline. Hemidesmosomes (hd) can be seen at the base, but the cells are separated by a space (arrow) with no gap junction closing off the intercellular space. The intercellular space is widened and partially filled with amorphous material (star).

X 45,800



The fibril bundles and individual fibrils are no longer as parallel to the base of the epidermis. There is some variation in the diameter of individual collagen fibrils and density of packing of the fibrils. In addition to an increased number of fibroblasts, some macrophages and other inflammatory cells can be seen in the dermis.

12 TO 15 WEEKS 3,4 BENZOPYRENE EXPOSURE: PAPILOMAS

A. General Organization

After 12 to 15 weeks of exposure to 3,4 benzopyrene the first papillomas appear, and by fifteen weeks all mice had developed tumors. These occur near the base of the pinna on the treated epidermal surface. These early tumors are slow growing, forming a crusty cutaneous horn on the surface. They do not spread under the adjacent skin, but appear to grow by expansion of a compact tumor mass. Several adjacent small papillomas often coalesce into a large one.

There is a smooth histological transition from the surrounding hypertrophied epidermis into the papilloma. This transition consists of a gradual increase in cell layers. Wide papillae extend into the dermis. Adjacent hair follicles appear incorporated into the tumor and become indistinguishable from the wide papilla (Fig. 16). Sebaceous glands have completely atrophied and are no longer seen. These wide papillae are usually located toward the periphery of the tumors. The basal cells in these papillae are closely packed and regularly arranged. The boundary between these cells and the underlying connective tissue is easily distinguished (Fig. 16).

At the base of the main tumor mass, organized areas in which the dermo-epidermal junction is easily distinguished alternate with areas of cellular disorganization where the dermo-epidermal junction is not clearly delineated at the light microscopic level (Fig. 17). No tongues of cells or individual cells are seen spreading out from the main tumor into the surrounding connective tissue. Beneath the tumor the dermis has become even more cellular. This is especially true below those tumors in which necrotic tissue has caused an inflammatory reaction (Fig. 18).

In some papillomas connective tissue spikes with blood vessels project into the tumor mass. In others this is not the case and the entire central portion of the papilloma may necrose (Fig. 18). In the former more vascularized tumors, there is a clear delineation between epidermal cells and connective tissue over a greater percentage of the tumor-connective tissue interface.

Within the tumor are whorls of keratinized material, areas of close cell to cell packing, areas of widened intercellular spaces, and occasional necrotic centers (Fig. 18). The tumor cells vary considerably in shape from one part of the tumor to another. In densely-packed areas with well-organized histology, cells are usually cuboidal or polygonal in shape. Near epithelial pearls, the cells appear dense and flat (Fig. 16). Cells that are more pleomorphic or generally round in shape occur in areas of the tumor in which the histology is disorganized at the light microscopic level. The intercellular spaces are widened and some cells appear to have pseudopodia-like

Figure 16. Papilloma. Near the edge of the papilloma there is a smooth transition from hyperplastic epidermis (left) into the main body of the papilloma (right). Wide papillae extend into the dermis (arrows) and hair follicles (HF) become incorporated into the papilloma. At the right is a keratin pearl (K). The cells are roughly of uniform size, and a condensation of connective tissue can be seen at the dermo-epidermal junction (star).

X 1800

Figure 17. Papilloma. At the base of the papilloma (P), areas where the dermo-epidermal junction is clearly delineated (1) alternate with areas where this is not clearly delineated (2). Most of the cells are roughly polygonal in shape, with only moderate variation in size.

X 1450

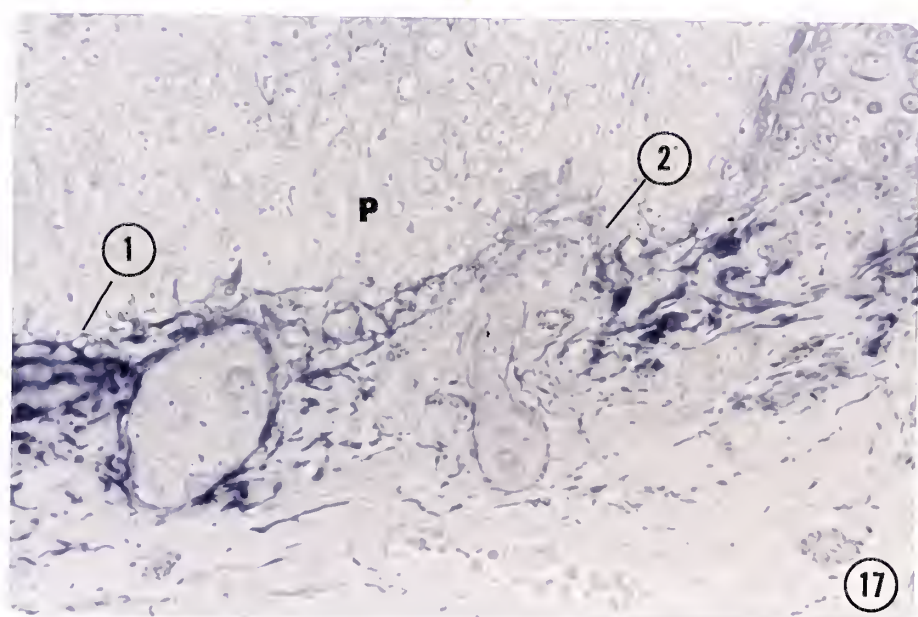
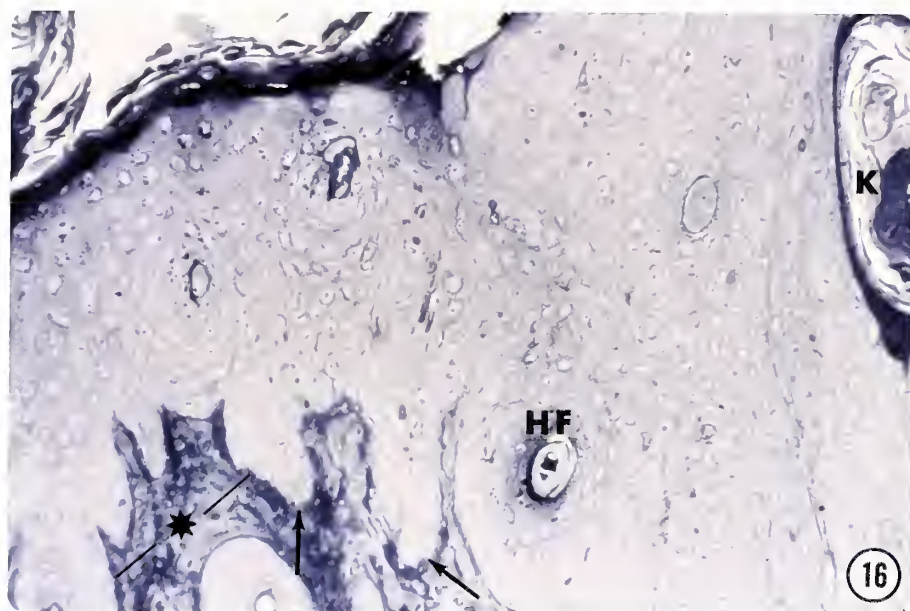
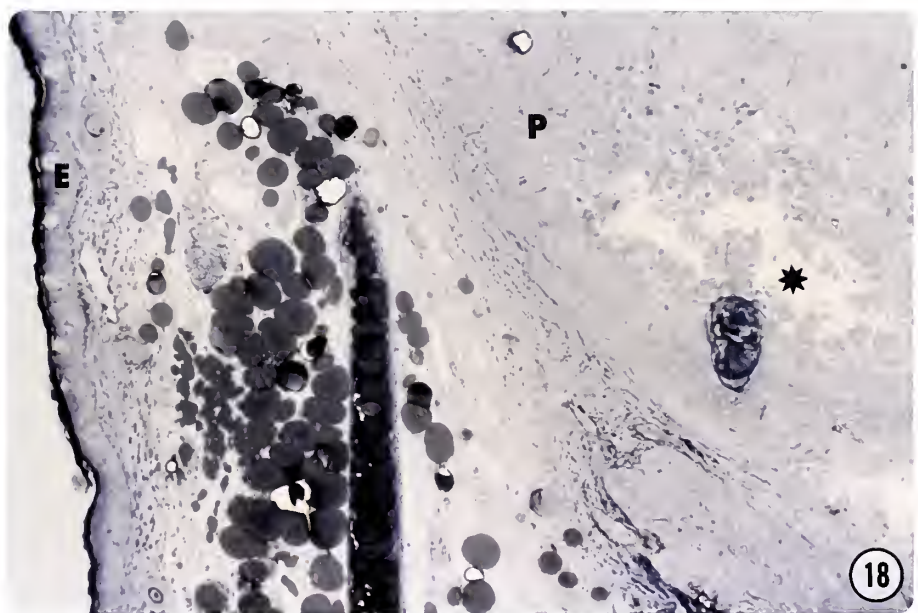


Figure 18. Papilloma. This papilloma (P) is poorly vascularized and an area of necrosis (star) can be seen. The growth pattern appears to be expansive rather than invasive in character. No narrow tongues of epithelial cells appear to infiltrate the surrounding tissue. On the opposite side of the pinna the normal epithelial surface (E) has undergone a mild hyperplasia.

X 460



processes extending to the adjacent cells.

B. Cytological Structures

1. Nucleus and Organelles

At the electron microscopic level, there is considerable variation in the structure of cells. Some cells are almost normal or comparable to those of the previous stage, while others show more extensive structural changes. The nucleus generally conforms to the shape of the cell, and in the few highly pleomorphic cells the nuclear contour is irregular. The heterochromatin is sparsely distributed at the periphery of the nucleus. One or two prominent nucleoli are eccentrically located in the nucleus (Fig. 19).

The hyaloplasm of many cells appears less electron dense than in normal epidermal cells. The tumor cells contain two or three large Golgi complexes adjacent to the nucleus (Fig. 19). These are often well developed with a large array of associated vesicles and vacuoles. There is a moderate to large amount of membranous material in the cytoplasm. The increased amount of vacuoles and dilated cisternae of rough-surfaced endoplasmic reticulum is similar to that seen in the focal areas of excessive hyperplasia present at six weeks of benzopyrene treatment.

Ribosomes are present in large numbers completely filling most cells and are organized into polysomes. Rarely a few cells occur in which the ribosomes are especially numerous and occur singly with no tendency to associate as polysomes (Fig. 20). These cells are generally pleomorphic in shape with large pseudopodial-like extensions. Mitochondria are present in

Figure 19. Papilloma. The cells from this area of the papilloma are rounded. Few desmosomes connect adjacent cells, and the intercellular space (star) is wide. A small number of microvilli (Mv) project into the intercellular space. Within the cells the nuclei (N) are round with an eccentrically placed nucleolus (n). Tonofibrils (T) are still abundant; they are arranged in a circular pattern around the nucleus. As many as three Golgi complexes (G) can be seen in one cell. The ribosomes are moderately abundant and are associated into clusters (polysomes). Within the matrix of some of the mitochondria a round dense body (db) can be seen. At the periphery of these cells are some fine 60 Å filaments (arrows).

X 14,200

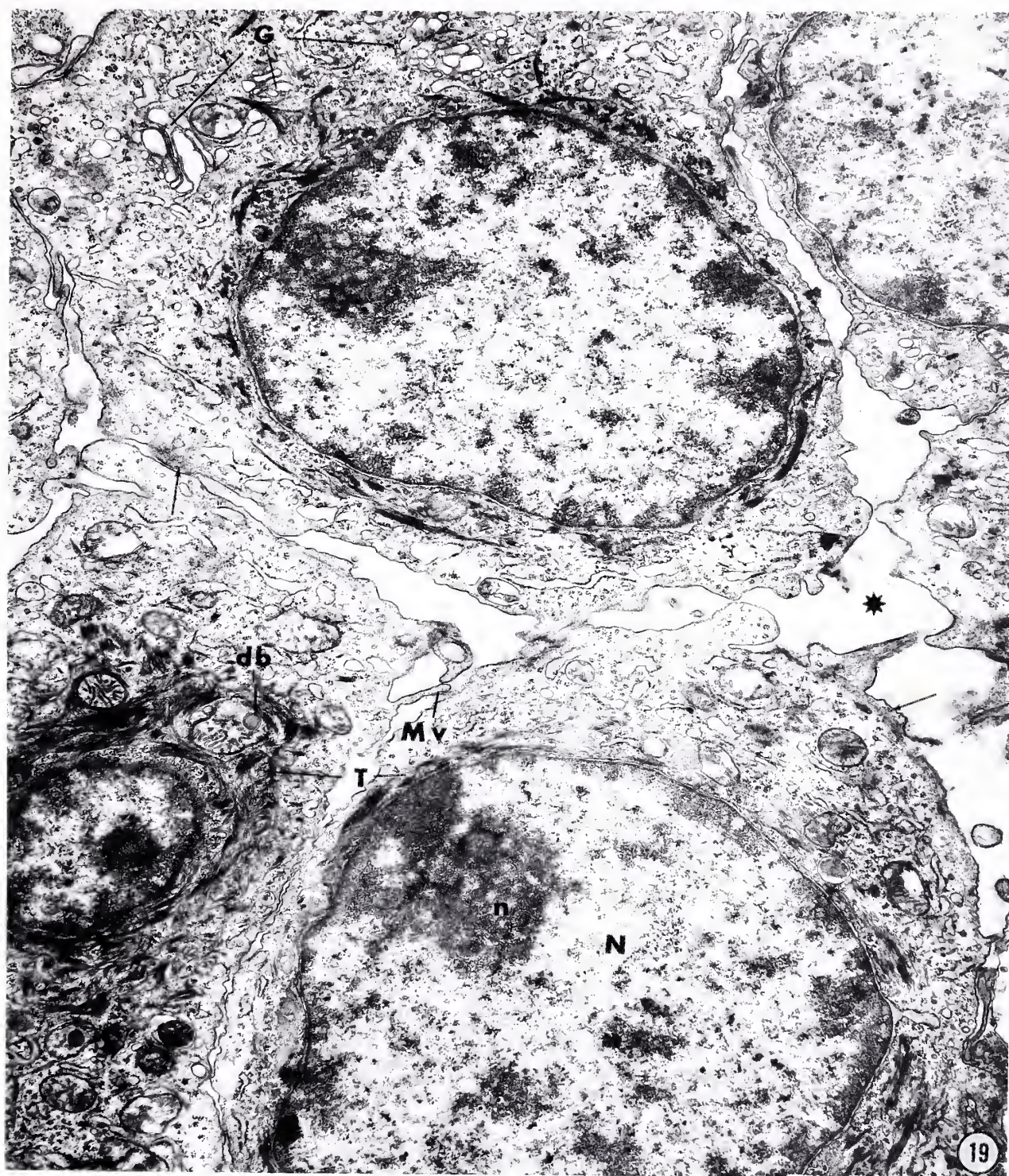
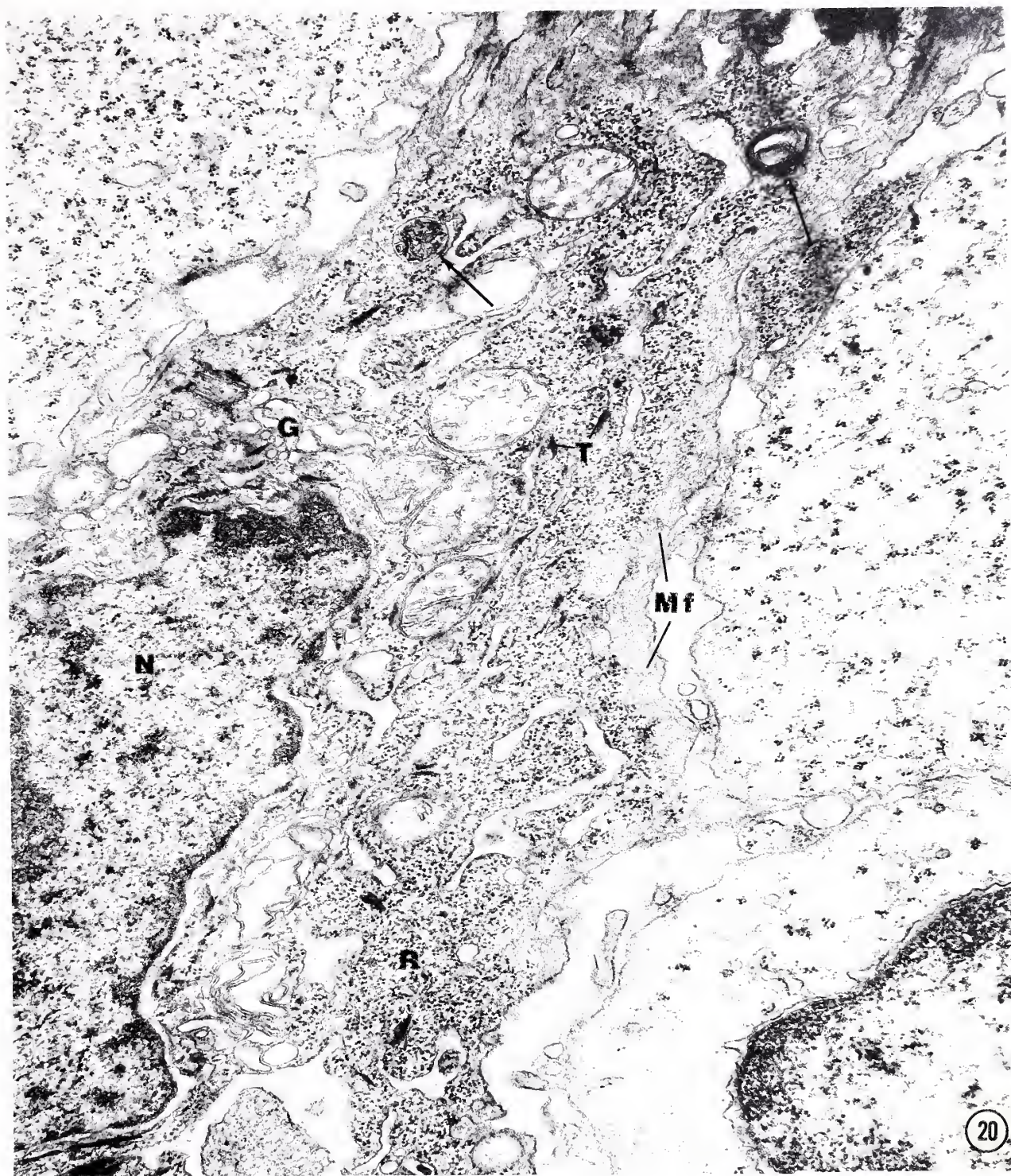


Figure 20. Papilloma. Rarely, a pleomorphic ribosome-rich cell can be seen. These cells contain a pleomorphic shaped nucleus (N), dilated Golgi (G), abundant free single ribosomes (R), and long cortical bands of 40 Å microfilaments (Mf). In addition, a myelin figure and other phagocytic vacuoles (arrows) can be seen. These cells are recognized as epithelial in origin only by the presence of short spike-like tonofibrils (T). The cells on either side of the pleomorphic cell contain few filaments and the ribosomes are clustered as polysomes.

X 27,400



moderate numbers in all cells. They generally resemble those described in the cells after six weeks of treatment. Some cells contain dense vacuoles resembling lysosomes, autophagic vacuoles, multivesicular bodies and myelin-like inclusions (Fig. 20).

2. Filaments

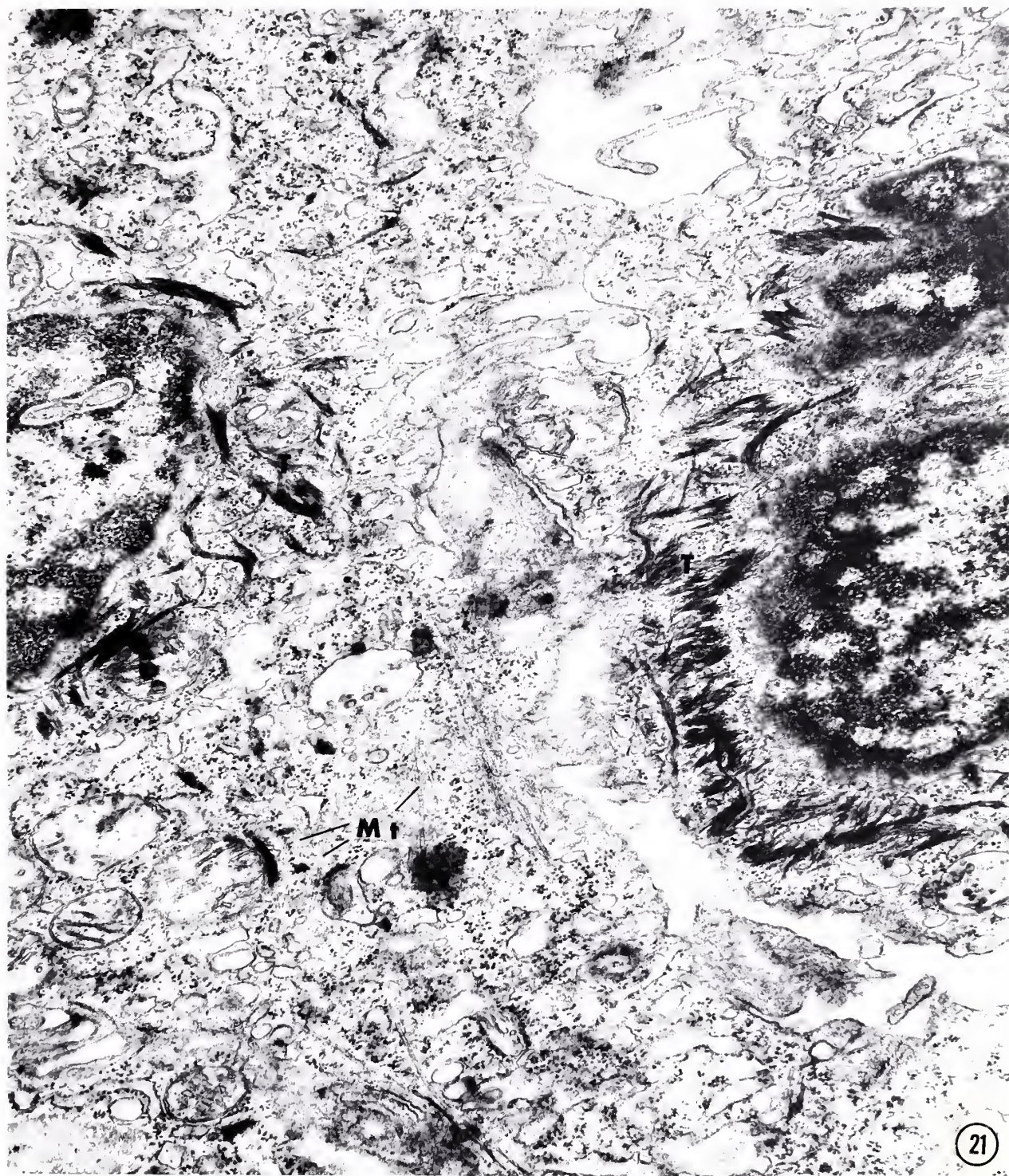
Tonofibrils are no longer as prominent in the epidermal tumor cells. Some cells, especially close to the epithelial pearls, may contain large arrays of tonofibrils. In most other cells of the tumor, tonofibril bundles appear in a circular orientation about the nucleus. Other short bundles can be seen scattered about the cytoplasm, arranged in random fashion (Fig. 19). A few cells may have a bizarre pattern such as a sunburst-like appearance with tonofibers arranged perpendicular to the nucleus (Fig. 21). The rare pleomorphic ribosome-rich cells contain few tonofibrils (Fig. 20). The fibrils are short and appear spike-shaped due to associated dense material. Although scanty, the presence of tonofibrils in these highly modified cells serves to indicate their epidermal origin.

Microtubules are present in most of the tumor cells (Fig. 21). They are randomly oriented, but may appear in clusters of three or four parallel to one another. They are as numerous as described in the six week treated epidermis.

The 40 Å microfilaments are more prominent in cells of the papilloma than at the hyperplastic stage. Many cells have 40 Å microfilaments in the small villi extending from the cell surfaces. In addition, they occasionally occur in small focal areas under the membrane (Fig. 19). Long cortical arrays of

Figure 21. Papilloma. These cells contain abundant tonofibrils (T). In the cell on the right the tonofibrils are arranged in a sunburst-like pattern around the nucleus. Many microtubules (Mt) occur in these cells.

X 30,200



microfilaments are only rarely seen and occur in the pleomorphic, ribosome-rich cells. Other cell organelles are excluded from these areas (Fig. 20).

In addition to the 40 Å filaments and tonofilaments, a third population of filaments is present at this time. These filaments are similar to the 40 Å filaments in that they are found at the cell periphery and extending into cytoplasmic processes. They differ from the 40 Å filaments in that they are slightly thicker (60 Å in diameter) and do not form a polygonal network (see Fig. 45). They pursue a straighter course, and are found deeper within the cytoplasm. Unlike the 40 Å microfilaments these filaments are not applied immediately adjacent to the plasma membrane, but may be separated from it by a narrow space that can contain vesicles or ribosomes. They form a long band parallel and close to the basal plasma membrane in some cells at the base of the tumor (Figs. 22,23). These cells generally border areas of dermal disorganization, and extend pseudopodia into the dermis. The pseudopodia have a narrow base and a ballooned-out tip. The 60 Å filaments may run across the base of the pseudopodia but do not actually enter them. 40 Å microfilaments may occur in these pseudopods but only in very small focal areas at the periphery.

3. Intercellular Relationships

As mentioned previously, the epidermal cells can be closely packed or separated by large intercellular spaces. Many microvilli and small pseudopodial extensions project into the intercellular space from the cell surface. In the more organized areas of the tumor, intercellular relationships are normal

Figure 22. Papilloma. At the base of the papilloma in some areas, the dermis (D) is devoid of collagen, and the basal epithelial cells project ballooned-out pseudopodia (p) into the dermis. These pseudopodia appear to contain some ribosome-like particles and some amorphous material at the periphery. Basement lamina has almost disappeared, occurring only as focal patches (arrow) under hemidesmosomes. Within these cells is a band of 60 Å filaments (f) running parallel to the base of the cell. Tonofibrils (T), polyribosomes (R), microtubules (Mt), and mitochondria (M) can also be seen. Many small vesicles (v) also occur.

X 28,600

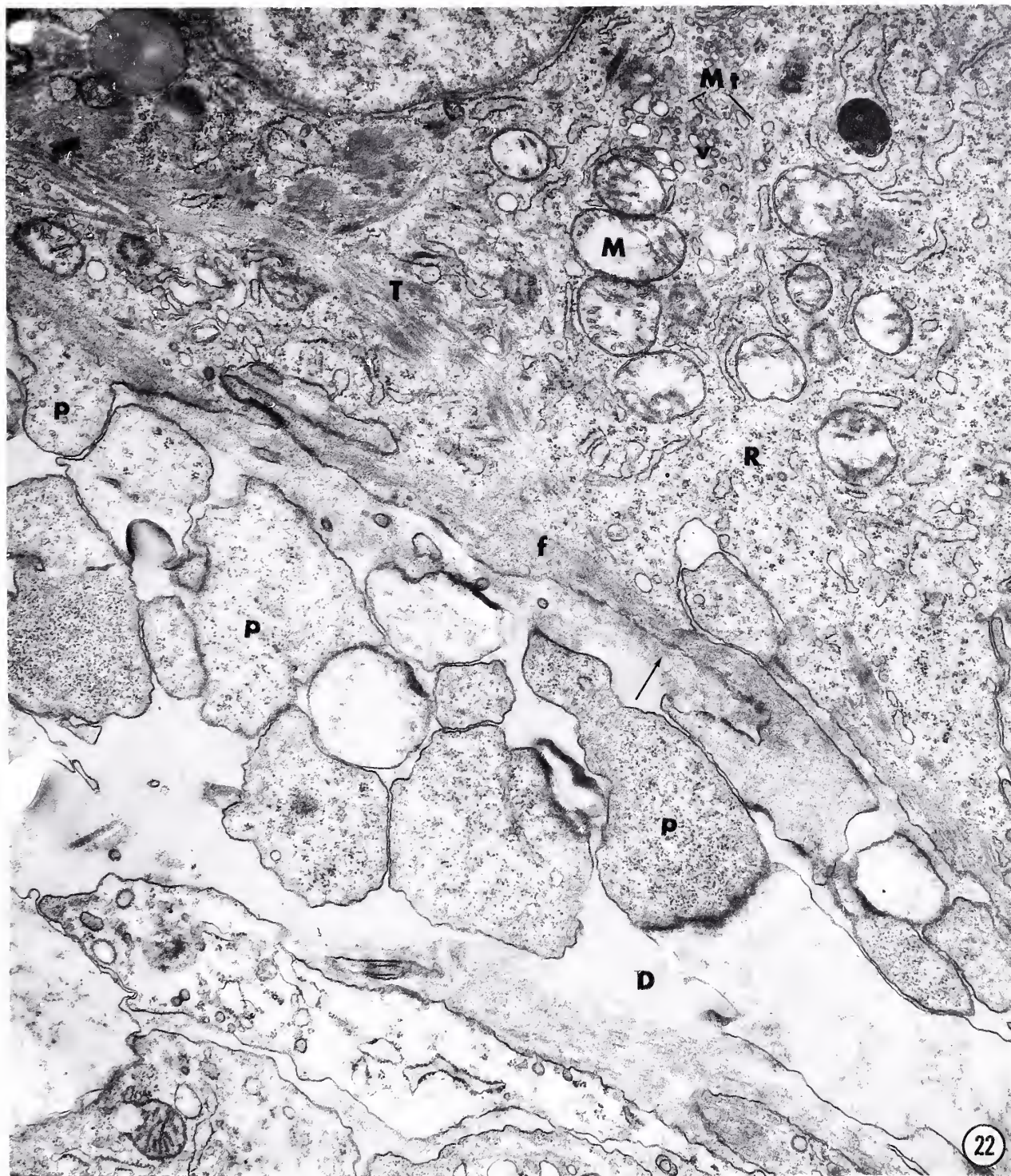
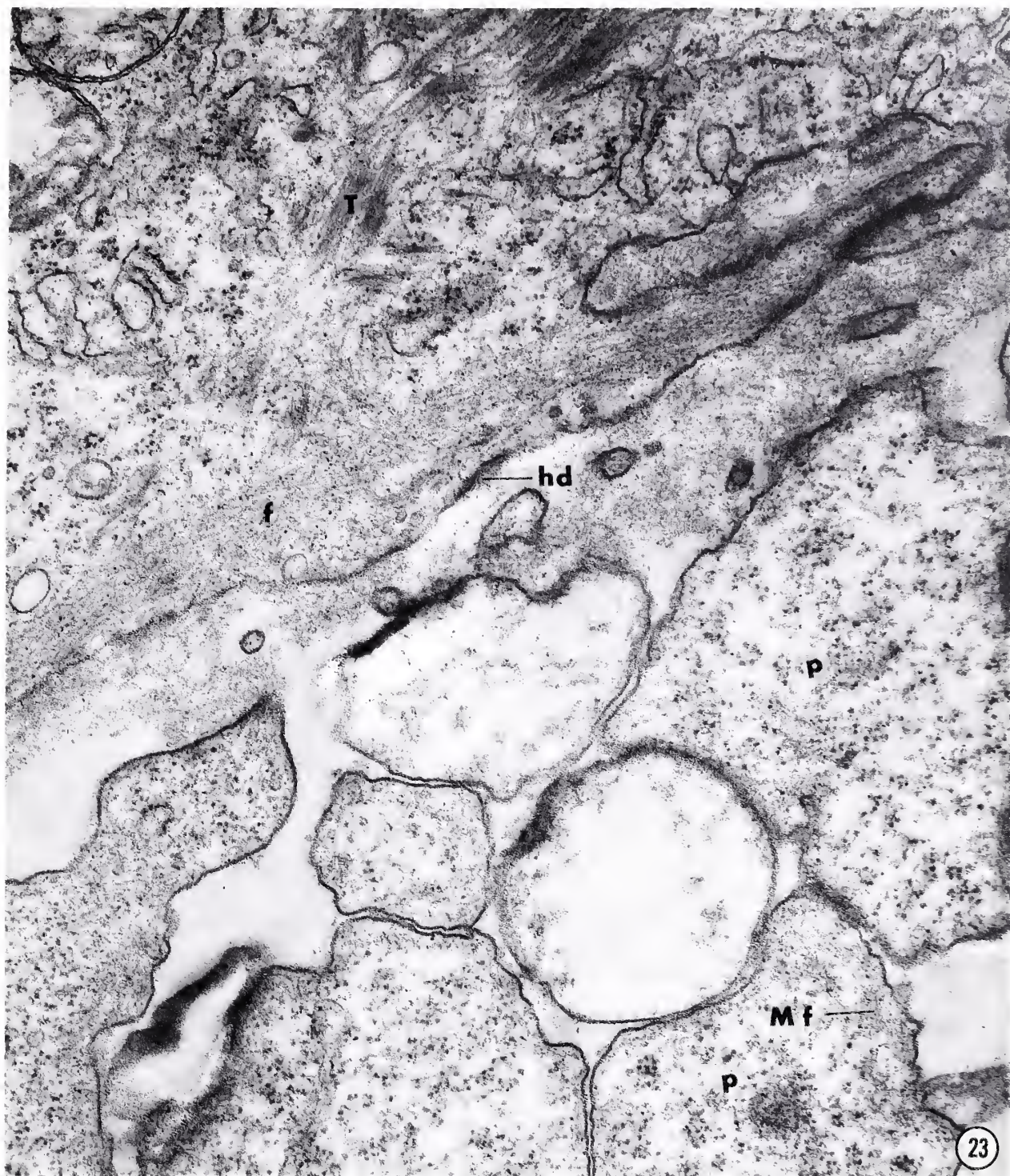


Figure 23. Higher magnification of Figure 22. The ballooned-out pseudopodia (p) contain ribosome-like particles and an occasional small bundle of 40 Å microfilaments (Mf). No hemidesmosomes (hd) occur on these pseudopodia, although some can be seen at the base of the epidermis. A basement lamina cannot be distinguished. Within the cell 60 Å filaments (f) run parallel to the base. Tonofibrils (T) can be seen in which some of the individual tonofilaments are apparent.

X 47,200



with 150 to 300 $\text{\AA}$ intercellular spaces between polygonal shaped cells (Fig. 22). In less organized areas of the papilloma, especially where the intercellular spaces are widened, desmosomes are scarce (Fig. 19). Other junctions such as gap and tight junctions are occasionally seen between cellular processes. A gap junction does not always seal off the intercellular space at the base.

Hemidesmosomes occur in normal numbers or with only slightly decreased frequency at the base of the tumor. However, many of them do not have a thick dense plaque or tonofibril insertion associated with the inner side of the membrane. They are recognized as hemidesmosomes only by the short thin extracellular dense plaque located about 40 $\text{\AA}$ below the plasma membrane. Hemidesmosomes do not occur on the ballooned-out pseudopodial extensions of basal cells into the connective tissue (Fig. 23).

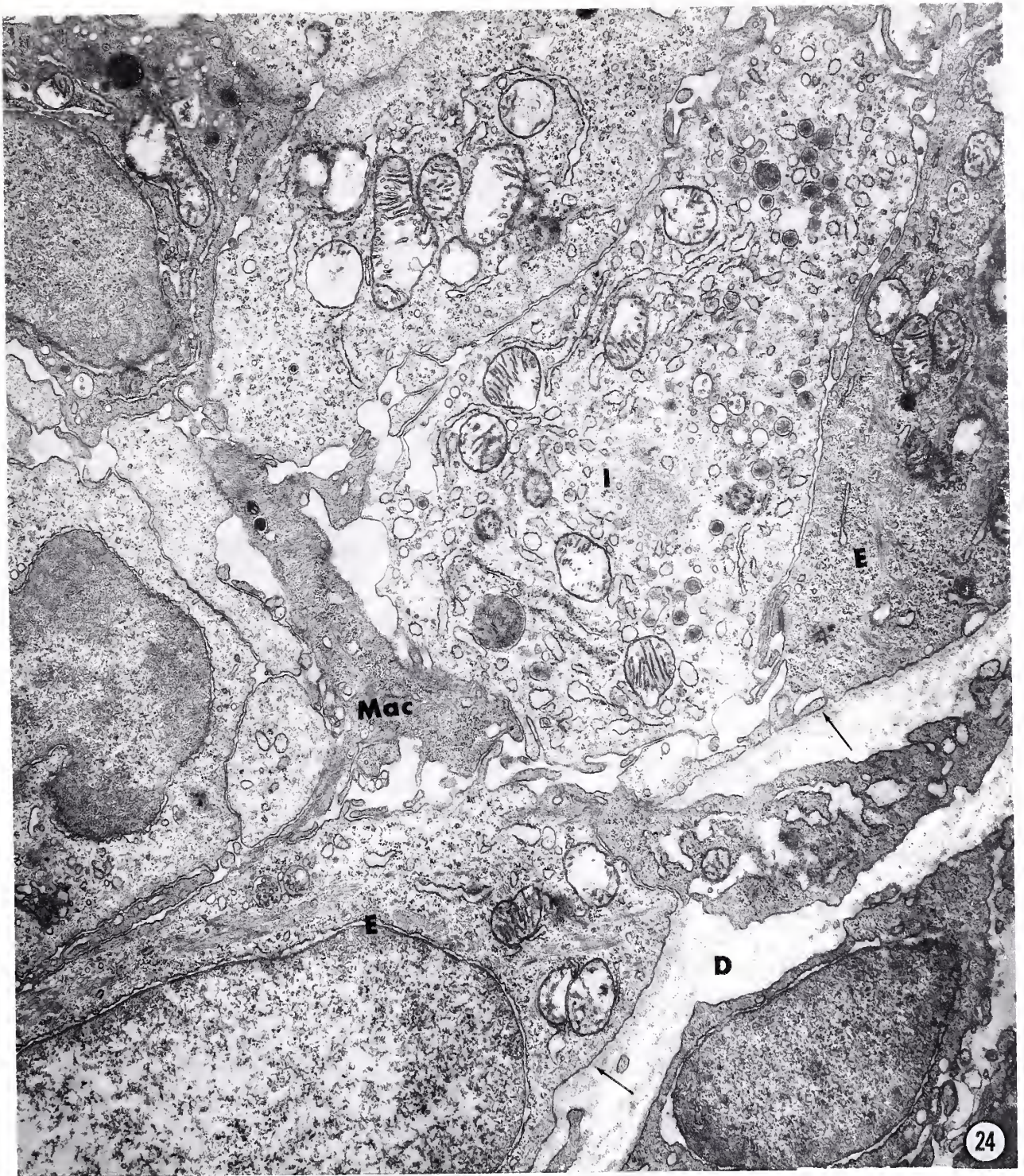
4. Basement Lamina

A basement lamina underlies most of the epidermal tumor cells adjacent to connective tissue. In focal areas of the tumor the basement lamina is fragmented with short areas either missing or very electron lucent. It is in the areas of basement lamina fragmentation that the basal cells send out ballooned-out pseudopodia through the basement lamina into the connective tissue (Figs. 22,23).

These pseudopodia may contain an occasional vesicle, a small number of free ribosomes and a few microfilaments under the membrane. No basement lamina surrounds these pseudopodia (Fig. 23).

Figure 24. Papilloma, low magnification. The dermis (D) here is less disrupted, and a basement lamina (arrow) can be seen underlying the epidermal cells (E). Inflammatory cells (I) can be seen within the tumor, and a macrophage (Mac) can be seen to breach the basement lamina and migrate between epidermal cells.

X 13,900



C. Dermis

In some areas beneath the papilloma, the dermal collagen architecture is no longer apparent. Fibroblasts and inflammatory cells are common in these areas of the dermis, and are surrounded by either an amorphous material or no discernible extracellular material at all. Only occasional strands of collagen filaments can be discerned (Figs. 22,23). Beneath more organized areas of the tumor the collagen fibrils are disorganized and wavy, but no empty spaces or dissolution of collagen is apparent. Occasionally a macrophage or other inflammatory cell appears to breach the basement lamina and migrate between the epidermal cells (Fig. 24).

20 TO 24 WEEKS 3,4 BENZOPYRENE EXPOSURE: CARCINOMAS

A. General Organization

Between twenty and twenty-four weeks of continuous exposure to 3,4 benzopyrene, some of larger papillomas have spread to the deep connective and muscular tissues of the head. New tumors develop in the hyperplastic epidermis of the pinna and grow by spread under the adjacent skin (Fig. 25). Some of these also rapidly spread to the underlying connective tissue of the head.

Unlike the early papillomas described previously, at the light microscopic level there is a sharp transition zone between these invasive tumors and the adjacent hyperplastic epidermis (Fig. 26). The pattern of tumor cell organization and relationships is highly distorted and irregular, suggestive of spread and infiltration of the tumor. The boundary between the original

Figure 25. Carcinoma. An invasive squamous cell carcinoma (C) can be seen to underlie normal hyperplastic epidermis (E). The tumor cells are variable in their staining properties, and quite variable in size and shape. A few large keratin pearls (K) can be seen within the carcinoma.

X 460

Figure 26. Carcinoma. Higher magnification of carcinoma cells (C) underlying normal hyperplastic epidermis (E). A blood vessel (v) is seen in the center of the picture. The normal epidermis is separated from it by connective tissue (star) while the carcinoma cells are separated from the vessel lumen only by the endothelial cells (arrow). The carcinoma cells are variable in size and shape. There is no apparent orientation of the cells and intercellular spaces are widened. The normal epidermal cells on the other hand are oriented perpendicular to the base and are roughly similar in size and shape.

X 1800

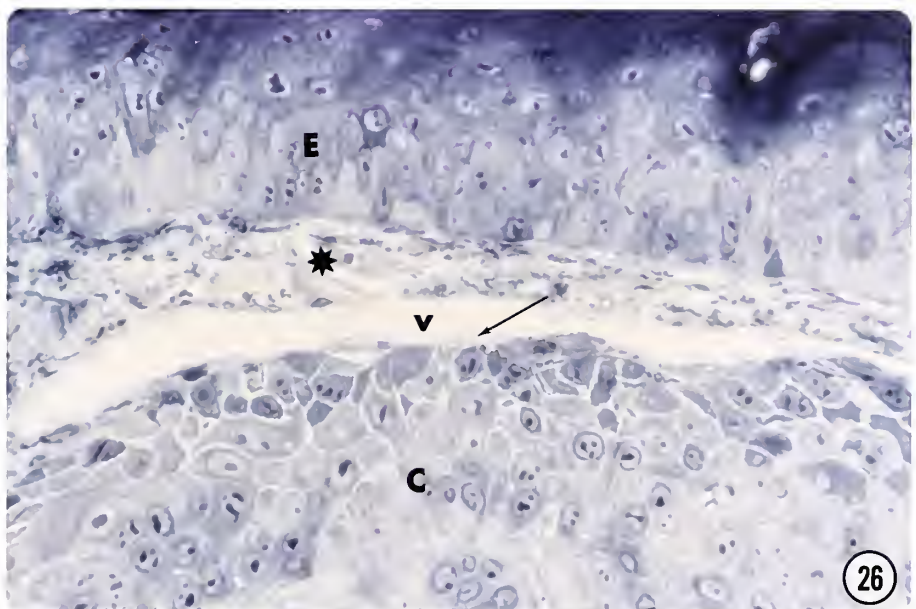
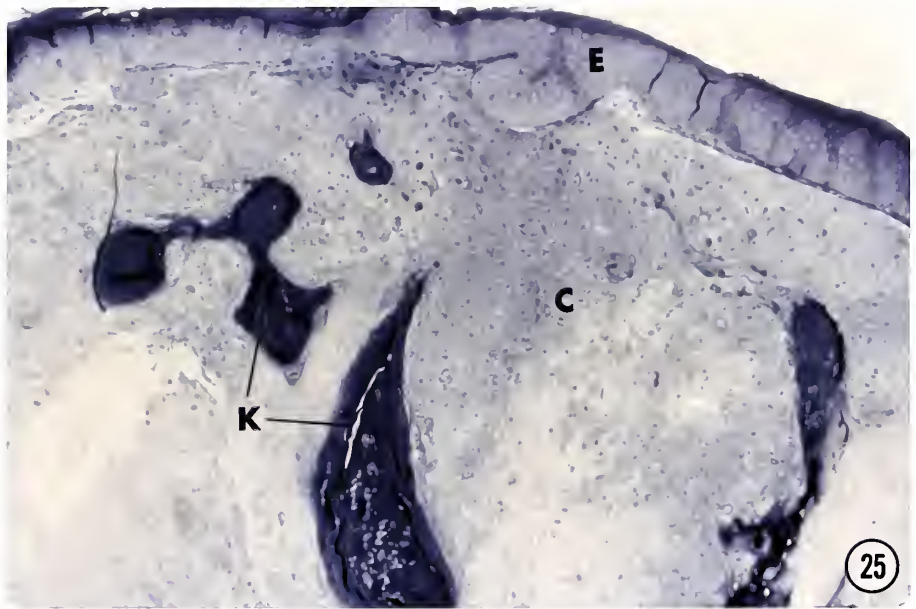
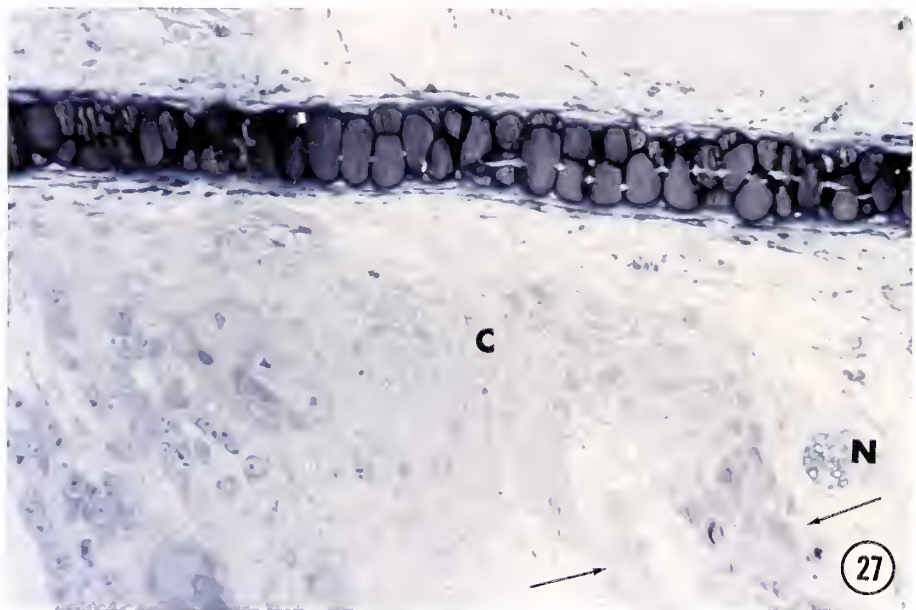


Figure 27. Carcinoma. The carcinoma cells (C) have invaded the deep layers of the connective tissue down to the central layer of cartilage. Tongues of carcinoma cells (arrow) directly invade the connective tissue and are about to surround a nerve bundle (N). The exact boundary between carcinoma and connective tissue is indistinct in many places.

X 1140



epidermis and underlying connective tissue is no longer readily evident in many areas. From the main tumor mass, tongues of carcinoma cells project into the subcutaneous tissue. Within the latter, large groups of epithelial cells, epithelial pearls, and cords of cells are interspersed among fibroblasts, macrophages, adipose cells, other connective tissue elements, and even surrounding the more deeply situated muscle cells and nerve sheaths (Fig. 27). Tumor cells can be seen under non-transformed hyperplastic epidermis separated by only a thin layer of connective tissue (Fig. 25). Tumor cells are also seen directly adjacent to the endothelial cells of capillaries and veins with no intervening connective tissue (Fig. 26). These gross and histologic characteristics are interpreted as transformation to squamous cell carcinoma. No other kinds of cancers are seen in these mice during the period of observation.

Within the cancers several distinct histological and cytological patterns are observed. One pattern already described is the keratinized epithelial pearl. The cytological characteristics and intercellular relationships are similar to those observed in the papillomas. The second pattern involves cells that are relatively polygonal or round in shape. With minor variations in cytology these cells resemble the majority of cells making up the papillomas of the previous stage (Fig. 28). As in normal epidermis both dark and light cells are present. The third pattern consists of the pleomorphic ribosome-rich tonofibril-deficient cells with pseudopodial extensions, seen in small numbers in the papillomas (Fig. 29). In the carcinomas they occur in large groups, especially in the leading edges of

the tongues of tumor infiltration. Thus, one major difference between the papillomas and carcinomas lies in the great increase in these pleomorphic, relatively undifferentiated cells, accompanied by increasing histological disorganization.

B. Cytological Structures

1. Nucleus and Organelles

The two cell types referred to above present differing cytological patterns. The polygonal, closely-packed cells appear to be more differentiated, containing more tonofibrils (Figs. 28,31,34). Their nuclei are generally round with an eccentrically placed nucleolus. The Golgi complexes are of the same appearance as in the papilloma. Ribosomes are numerous but tightly associated into polysomes. There is a moderate amount of rough-surfaced endoplasmic reticulum. Mitochondria appear similar to those in papilloma cells. Some contain dense round bodies in the matrix.

The less differentiated pleomorphic cells contain a nucleus that may be round or very irregular in outline. The heterochromatin may be more abundant and dense. A nucleolus is usually present. One or two Golgi complexes occur with a number of large cisternae that are often irregular in shape and dilated. Vesicles and vacuoles are scattered about the cytoplasm. Occasional multivesicular bodies and myelin figures are seen. Ribosomes are very numerous in these cells, but occur singly or only loosely associated as polysomes. Many of these cells contain a moderate amount of rough-surfaced endoplasmic reticulum, the cisternae of which are mildly dilated, containing some dark

Figure 28. Carcinoma. These cells are polygonal in shape. Dark cells and light cells (on right) can be distinguished. The intercellular space (star) is slightly widened, but not dilated into large lacunae. Desmosomes (d) are decreased in number. The nucleus (lower left) is large and round. In the cytoplasm, tonofibrils are decreased. Some may be seen to insert into the desmosomes (arrow). The cells contain abundant polyribosomes (R), rough-surfaced endoplasmic reticulum (ER), and bundles of 60 Å filaments (f).

X 14,100

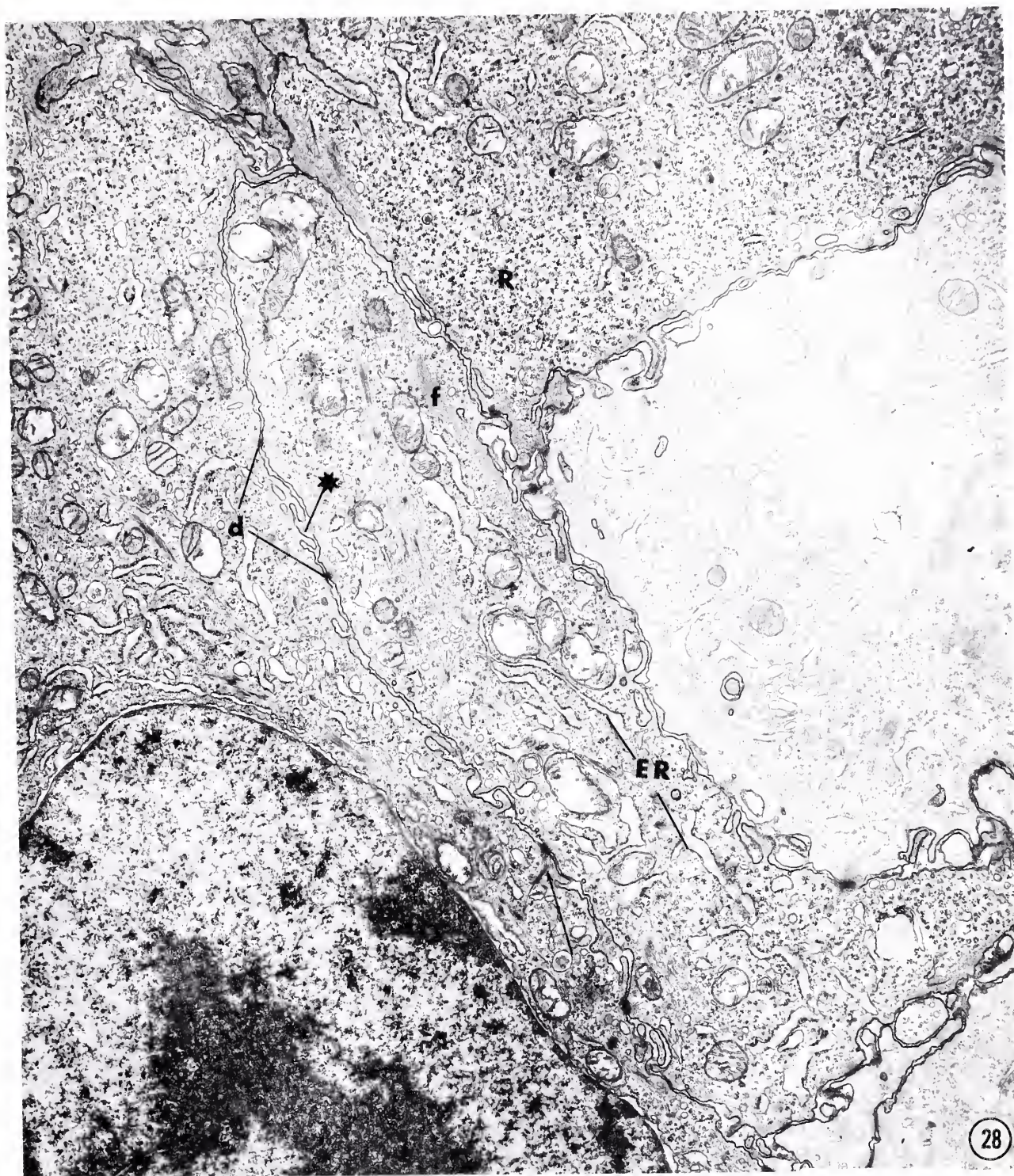


Figure 29. Carcinoma. These cells are more pleomorphic in shape. Many microvilli (Mv) extend into the extremely dilated intercellular space (star). The cells contain abundant free single ribosomes (R), short spike-like tonofibrils (T), and long cortical bands of 40 Å microfilaments (Mf).

X 29,700

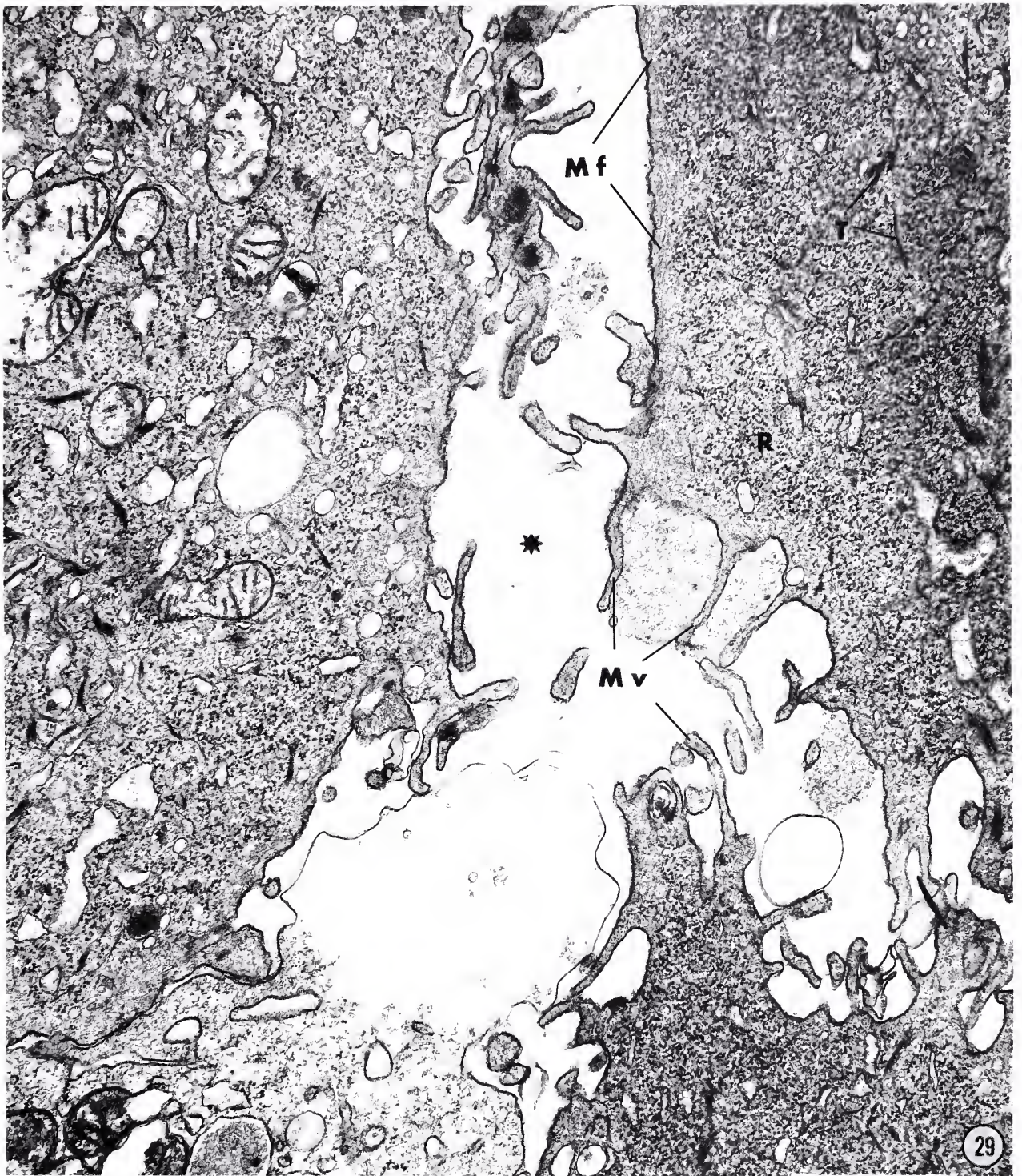
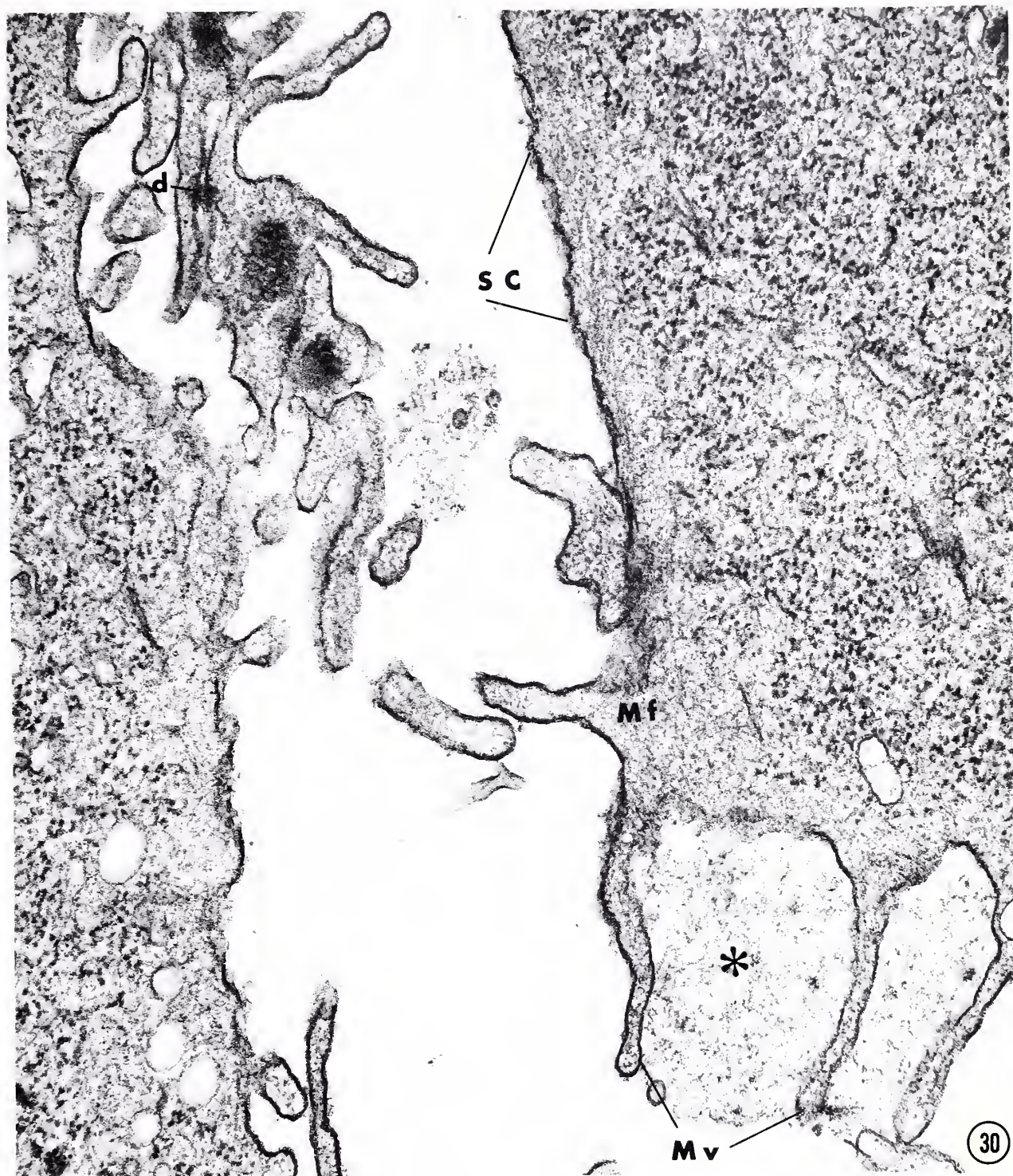


Figure 30. Higher magnification of Figure 29. The microvilli (Mv) are clearly seen extending into the empty intercellular space. Some amorphous material (asterix) adheres to the cells. On the cell surfaces an electron-dense surface coat (SC) can be distinguished. An occasional small desmosome (d) occurs on some of the villous extensions. Within the cell the cortical band of $40 \text{ \AA}$ microfilaments (Mf) is clearly distinguished as a polygonal network, extending under the plasma membrane into the microvilli.

X 63,700



flocculent material. The mitochondria appear similar to those in the more differentiated cells (Figs. 29,30,32,35).

2. Filaments

The polygonal, closely-packed cells contain moderate amounts of long tonofibrils. The pattern of distribution may be quite normal or bizarre as seen in the papillomas. Microtubules are very infrequently seen in these cells. The 40 $\text{\AA}$ microfilaments are only rarely seen in these cells other than in microvillous processes. The straight thick bands of 60 $\text{\AA}$ filaments have become more numerous (Figs. 28,31,34).

In the pleomorphic cells the tonofibrils have been reduced to a few short electron-dense spike-like structures (Figs. 29,30). These scanty tonofibrils are often the only means of determining the cell's epithelial origin. 40 $\text{\AA}$ microfilaments fill most of the cortex of these cells. These filaments may take slightly different appearances from cell to cell, probably due in part to the plane of sectioning. In many cells, a 500 $\text{\AA}$ thick region of the cortex is filled with a dense uniform meshwork of filaments in which at higher magnification a pattern of uniform and delicate array of polygons is seen (especially well seen in Fig. 42). 60 $\text{\AA}$ filaments are rarely seen (Fig. 45). Microtubules can be seen randomly oriented in some of these cells (Fig. 38).

3. Intercellular Relationships

The more differentiated polygonal cells are separated by an intercellular space that is irregular in size but usually less than 400 $\text{\AA}$ to 500 $\text{\AA}$ (Fig. 28). Microvilli are common at the surface, but are bent or coiled up in the relatively narrow

Figure 31. Carcinoma; more differentiated area of the tumor at the tumor-connective tissue junction. The epithelial cell (E) can be seen on the right. Within the connective tissue is a fibroblast (F) and an inflammatory cell (I). The extracellular areas of the dermis are filled with an amorphous flocculent matrix in which are embedded collagen fibrils (C). Beneath the epidermal cell are patches of basement lamina (arrows). The base of the tumor cell is irregular, but no ballooned out pseudopodia project into the connective tissue. Within the epithelial cell are basal bundles of 60 Å filaments (f), tonofibrils (T), free polyribosomes (R), rough-surfaced endoplasmic reticulum (ER), and irregularly shaped mitochondria (M).

X 14,300

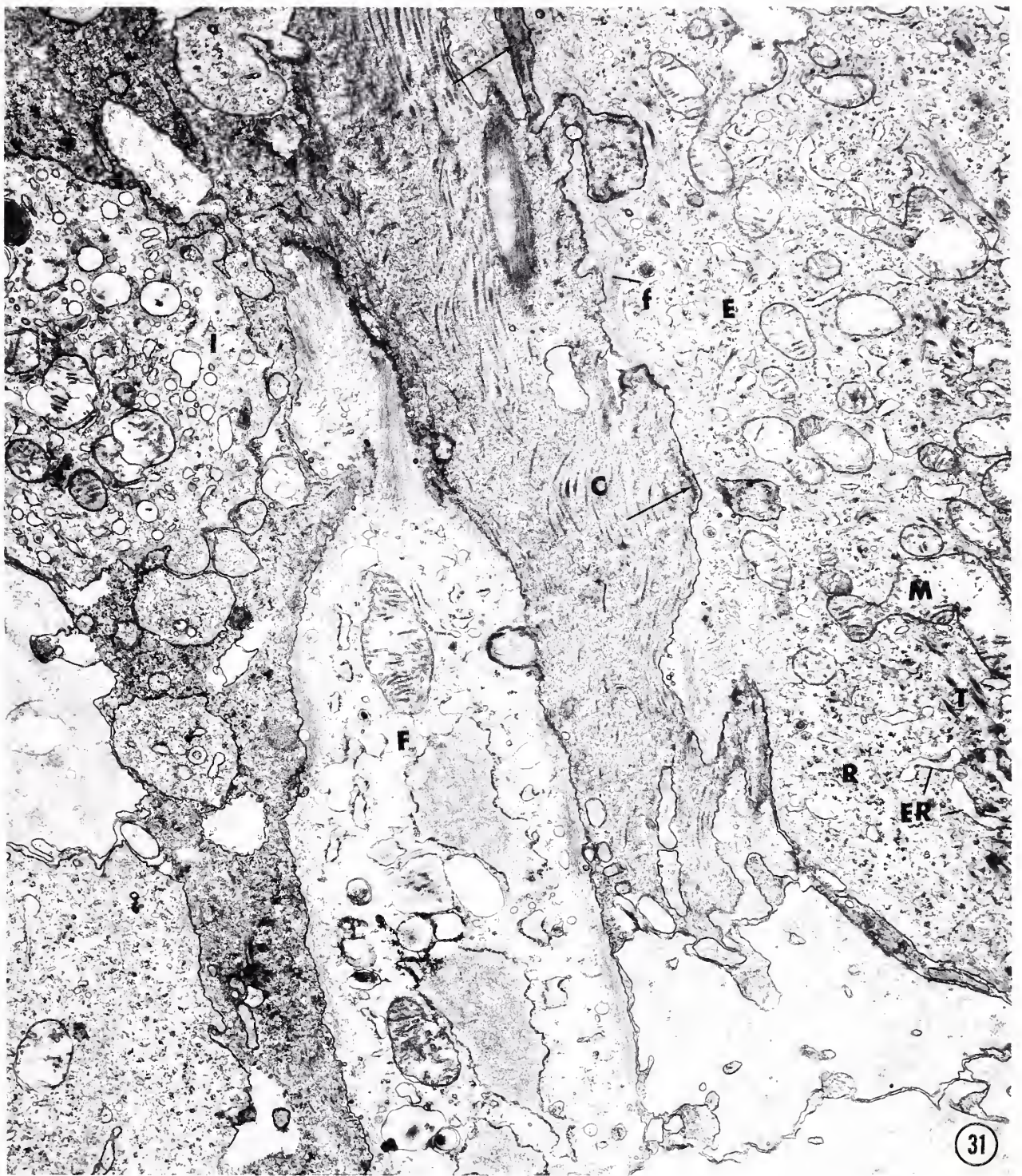
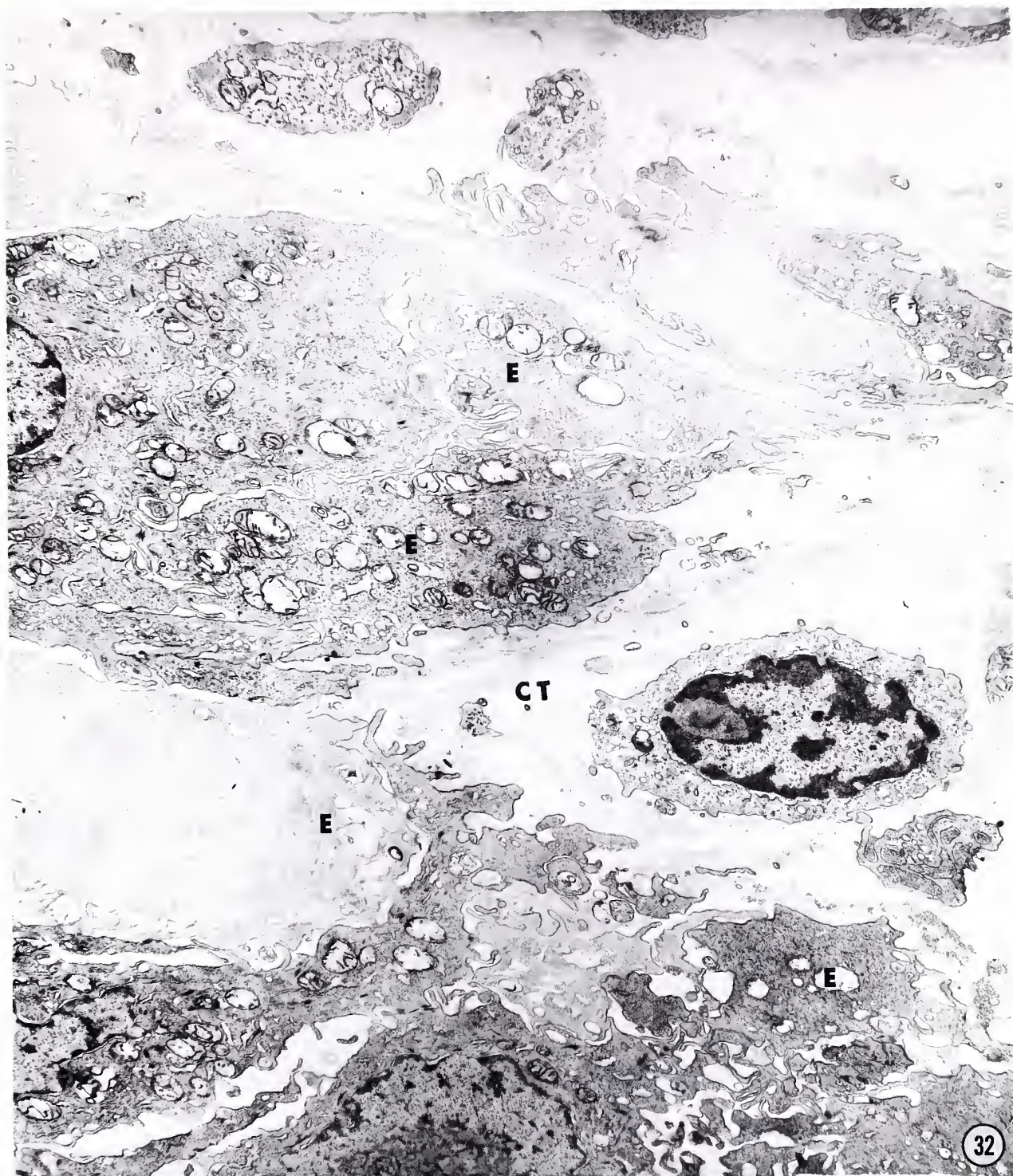


Figure 32. Carcinoma: less differentiated area of the tumor at the connective tissue-tumor junction. The epithelial cells (E) are pleomorphic in shape with many microvillous processes extending into the intercellular spaces and into the electron-lucent connective tissue (CT). No basement lamina is seen beneath these cells.

X 8,500



intercellular spaces (Fig. 33). Desmosomes are markedly reduced as well as tight and gap junctions, although no absolute quantitative estimate was made. Hemidesmosomes are slightly decreased in number.

The intercellular spacing between the less differentiated pleomorphic cells is invariably large, with many villous processes extending both from the main cell body as well as the major pseudopodial extensions (Figs. 29,32). These latter pseudopodia are large and tapered and contain many 40 Å microfilaments. Within the intercellular spaces adherent to these cells are large clumps of electron dense amorphous material. This material also appears to form an irregular thin layer on the outer surface of these cells. An occasional desmosome connects the tips of adjacent cells' villous processes. Usually, however, these cells are devoid of desmosomes or other specialized cell to cell attachments. A reduced number of hemidesmosomes do occur at the connective tissue junction of these cells. These are very inconspicuous and recognized only by the thin electron dense extracellular plaque.

4. Basement Lamina

The base of the more differentiated polygonal cells may be relatively smooth or quite irregular with blunt cytoplasmic processes projecting into the connective tissue (Figs. 31,34). These processes may have hemidesmosomes and are not as large or extend as deeply as the ballooned-out pseudopodia beneath the papilloma cells. Very little of the latter type of pseudopodial activity occurs in the carcinomas. Basement lamina may be absent or in patches under these cells. The small patches of

Figure 33. Carcinoma. High magnification of microvilli (Mv) extending from more differentiated cells into the intercellular space (ICS). There is some amorphous material (star) in the intercellular space and a small amount of surface coat material (SC) adherent to the cells. Within the cell, beneath and within microvilli is some flocculent material (arrow) which does not have the appearance of true microfilaments. A desmosome (d) is also seen into which insert some tonofibrils (T).

X 60,900



Figure 34. Carcinoma; high magnification of tumor-connective tissue junction in a more differentiated area of the tumor. The connective tissue (CT) is electron-lucent. The epithelial cell occupies the upper part of the picture. Beneath the epithelial cell is a mass of flocculent material (star). Basement lamina is not readily apparent, but small areas of increased electron density (arrows) occur beneath some of the hemidesmosomes (hd) in a location suggestive of basement lamina formation. Within the cells are bundles of tonofibrils (T) composed of 100 Å tonofilaments. There is also a bundle of 60 Å filaments (f).

X 59,700

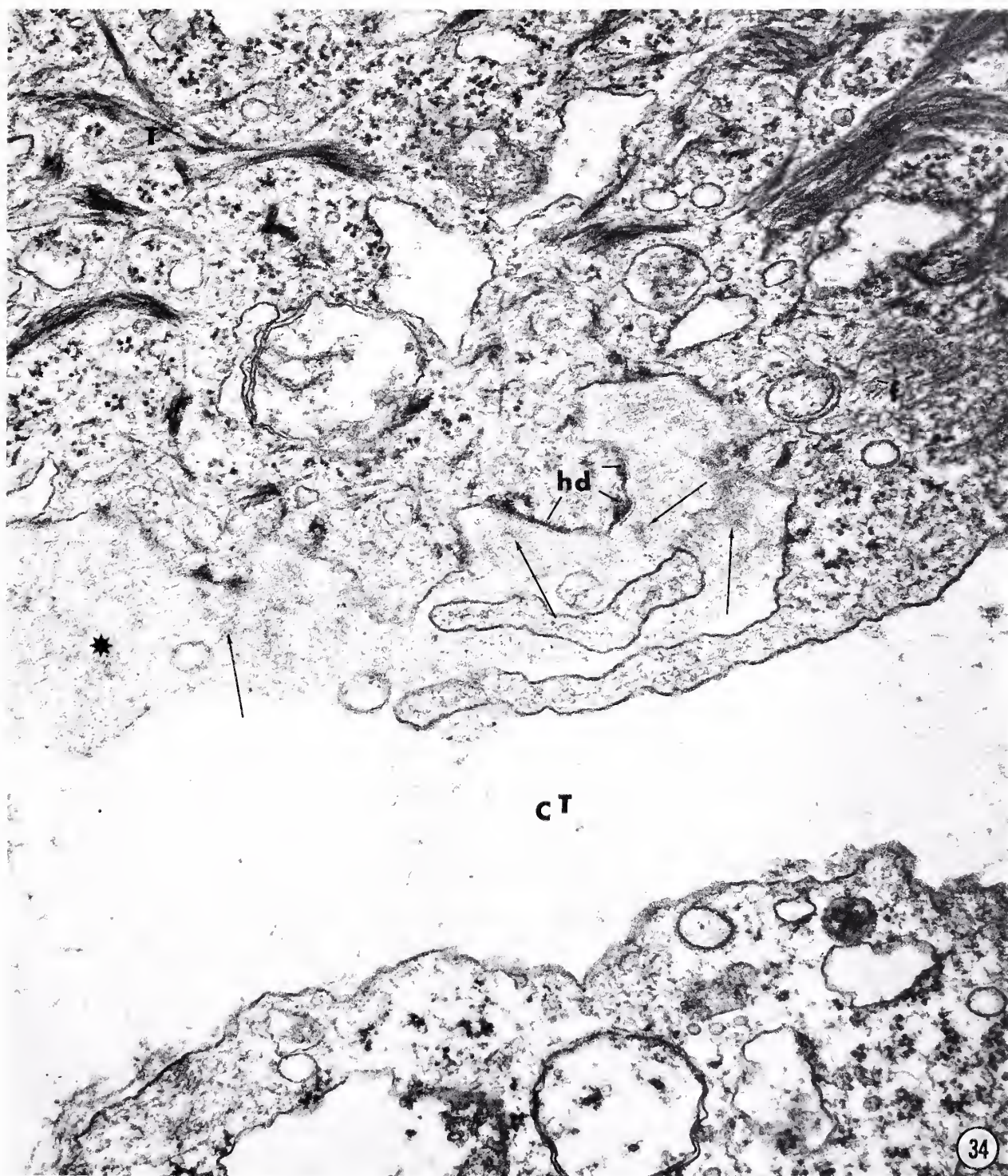


Figure 35. Carcinoma. High magnification of tumor-connective tissue junction in a less differentiated area of the tumor. The connective tissue is disrupted. Collagen fibrils (C) of various diameters embedded in a flocculent matrix alternate with electron-lucent areas. The epithelial cells (E) are very irregular in outline with many microvilli (Mv) extending into the connective tissue. No basement lamina or hemidesmosomes are seen. Within these cells, just beneath the plasma membrane a network of microfilaments occurs. Though no apparent orientation is seen, on close examination of certain areas (arrows) the polygonal web is discerned. (Compare with Figure 33.)

X 47,300



lamina are usually located beneath hemidesmosomes.

The undifferentiated pleomorphic cells extend major pseudopodia into the connective tissue. Beneath these cells basement lamina is rarely seen (Figs. 32,35).

C. Dermis

Connective tissue beneath the more differentiated cells is often relatively intact, with wavy bands of collagen fibrils embedded in an amorphous electron-dense matrix (Fig. 31). While beneath the pleomorphic undifferentiated cells the connective tissue is invariably disrupted, with electron-lucent areas alternating with electron-dense matrix containing a few randomly oriented collagen fibrils (Figs. 32,35).

LONG TERM EFFECTS OF 3,4 BENZOPYRENE EXPOSURE

By thirty-six weeks all mice treated with 3,4 benzopyrene had died by direct spread of the tumors to the head and neck. No metastasis to adjacent or distant lymph nodes or other organs were found. Two of the carcinomas were successfully transplanted to the flank of other mice of the same strain. One of the first generation transplants was again successfully transplanted. The histology and fine structure of these tumors appeared to be similar to the carcinomas described above.

CONTROL EXPERIMENTS

A. 24 Weeks 1,2,3,4 Dibenzanthracene Exposure

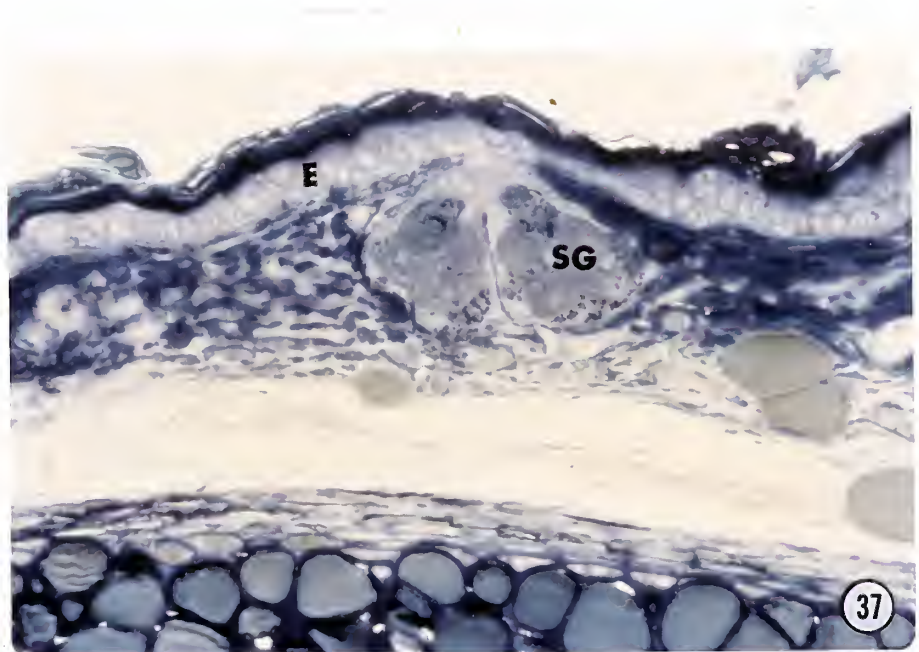
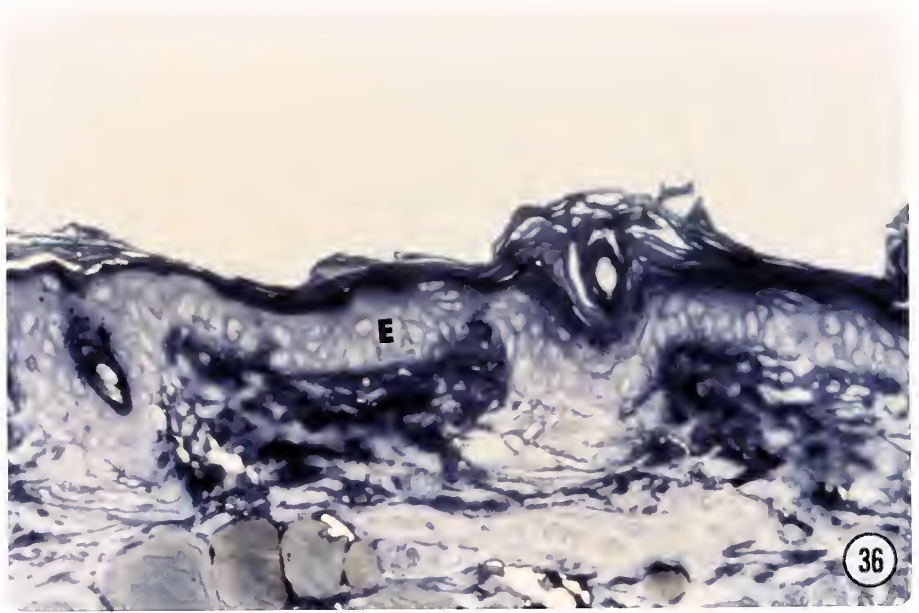
At twenty-four weeks of exposure to 1,2,3,4, dibenzanthracene the epidermis is hyperplastic, being two to three times

Figure 36. Control: eighteen weeks treatment with 1,2,3,4 dibenzanthracene. The epidermis (E) is only slightly hyperplastic, and no sebaceous glands are seen. Otherwise, there is little change from normal.

X 1800

Figure 37. Control: eighteen weeks treatment with acetone. The epidermis (E) is normal in thickness. Sebaceous glands (SG) are present and unaltered. There is no change from the normal untreated pinna seen in Figure 1.

X 1800



normal in thickness (Fig. 36). However, no focal areas of excessive hyperplasia or widened intercellular spaces are seen. No papillomas or carcinomas appear, even after observation for one year. Cell contact relationships and basement lamina are unaltered, and the cytology is almost indistinguishable from untreated epidermis. The dermis is unaltered, except that sebaceous glands have atrophied and are no longer seen.

B. 24 Weeks Acetone Exposure

Epidermis exposed to acetone for twenty-four weeks is indistinguishable from normal at the light and electron microscopic levels (Fig. 37). Cells are closely packed with many desmosomes and narrow intercellular spaces. The basement lamina is continuous and the dermal architecture is unaltered from normal.

CARCINOMAS EXPOSED TO CYTOCHALASIN B

In vitro treatment of thin slices of carcinoma tissue with 100 µg/ml of cytochalasin B and 0.4% dimethyl sulfoxide in mammalian tissue culture media results in morphological alteration of the 40 Å microfilaments and some cell processes. No other cellular structures were affected (Figs. 40,41,43,44). The 25 gm mouse injected intravenously with 800 µg of cytochalasin B in 0.1 ml dimethyl sulfoxide did not appear to suffer any ill effects in the hour prior to sacrificing the mouse for excision of the tumor. The morphological alterations were the same as those in vitro.

Exposure of carcinoma cells to cytochalasin B for one hour results in clumping of the long cortical bundles of micro-

filaments, thickening of the filaments, and the appearance of many small bead-like densities within the disrupted filamentous material (Figs. 41,43). In addition the fibrillar oriented appearance and polygonal structure of most of the microfilament bundles becomes less apparent. The cell surface is less irregular and the attenuated processes and microvilli usually seen on the untreated cells are blunted and reduced in number (Figs. 40,44).

The effects are most readily appreciated in the pleomorphic undifferentiated cells with long cortical bundles of microfilaments. The closely packed more differentiated cells do not seem to be affected as much.

The in vitro controls exposed to 0.4% dimethyl sulfoxide for one hour were indistinguishable from the carcinoma cells fixed immediately after excision. The microfilaments in these cells had the same delicate oriented fibrillar appearance at lower magnification (Fig. 38), and fine polygonal appearance at higher magnification (Figs. 39,42,45,46) seen in these cells fixed immediately after excision. The cell surface was irregular with many attenuated processes and microvilli.



Figure 38. Carcinoma. Control--0.4% dimethyl sulfoxide in vitro for one hour. This epithelial cell extension is irregular in outline with several long slender microvilli (Mv) projecting from the surface. No basement lamina is seen at the junction with the disrupted connective tissue (CT), though a thick surface coat (SC) can be seen on the cell surface. Within this cell many bundles of 40 $\text{\AA}$ microfilaments (Mf) occur at the periphery. Some are oriented in the long axis of this extension (arrows), while others form a delicate network with no particular orientation (star). Also within the cell are microtubules (Mt), free ribosomes (R), and rough-surfaced endoplasmic reticulum (ER). Tonofibrils (T) are very scarce, occurring only as a few very small bundles.

X 30,100

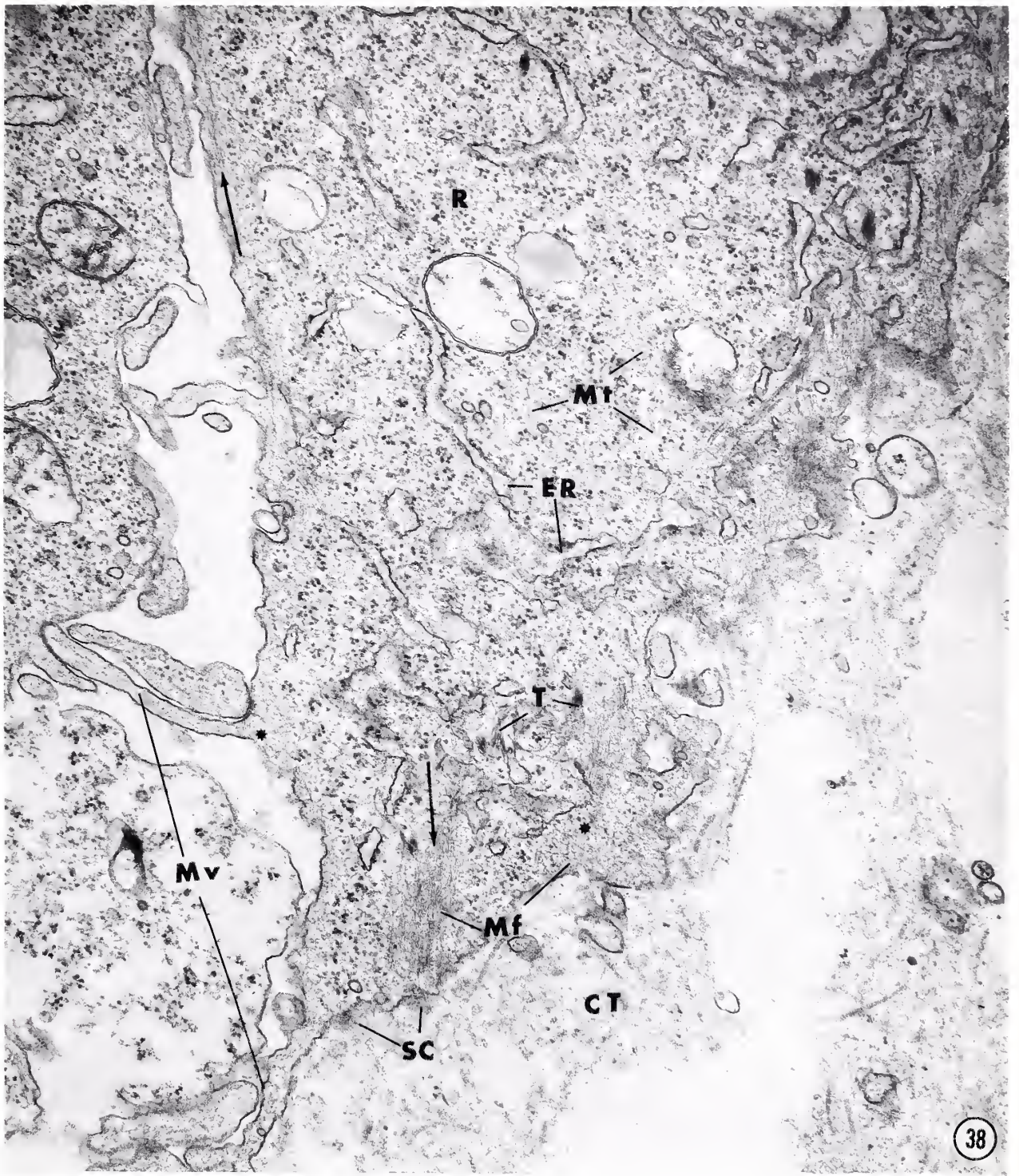


Figure 39. High magnification of Figure 38. The bundles of 40 Å microfilaments (Mf) can clearly be seen in some areas to consist of a regularly arranged network of polygons (arrows). The microvillous process (Mv) also contains microfilaments. The few short tonofibrils (T) composed of 100 Å tonofilaments seen in this cell define its epithelial origin.

X 64,000

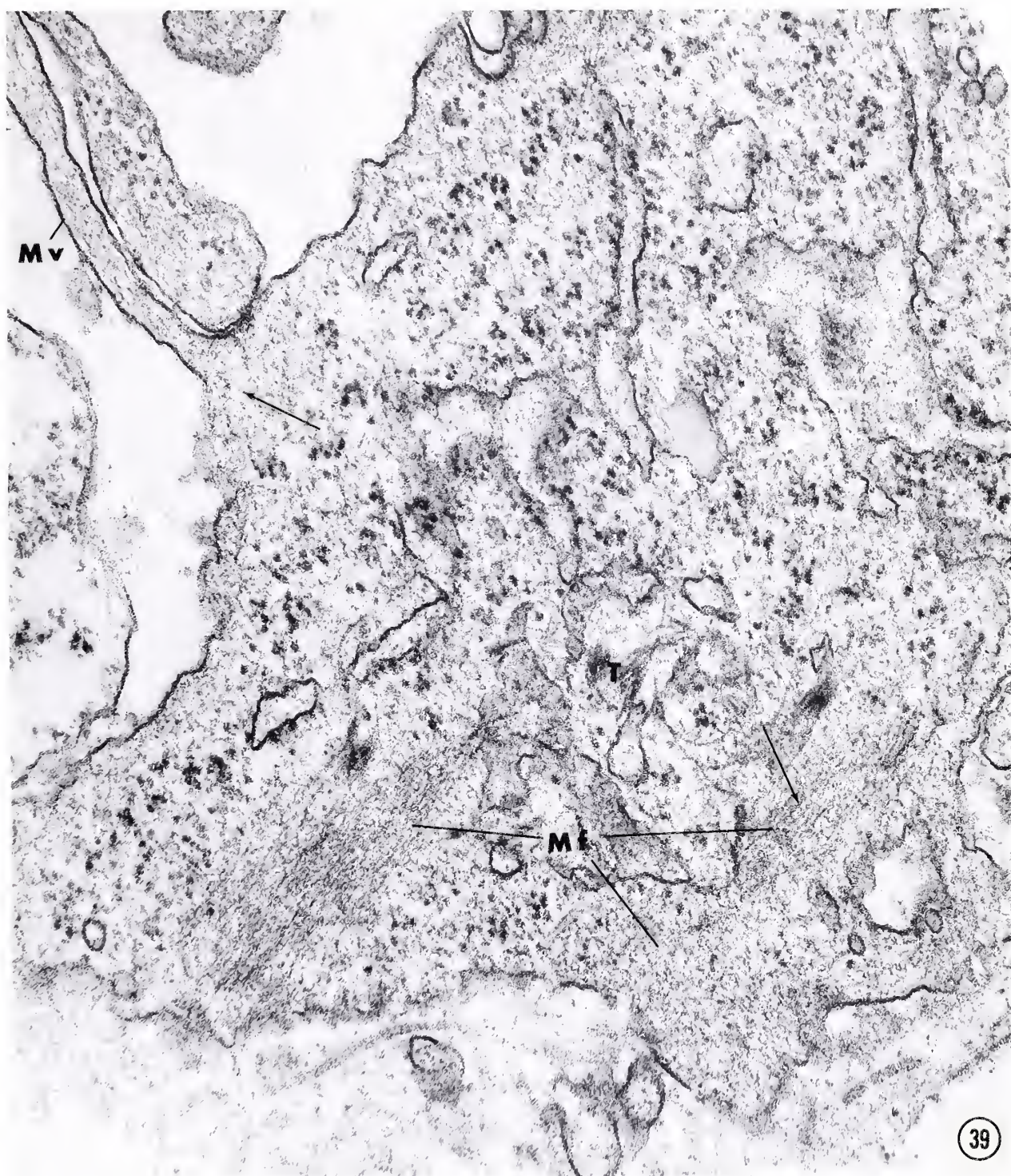


Figure 40. Cytochalasin B 100 $\mu\text{g/ml}$ in vitro for one hour. This cell extension is similar to that seen in Figure 38. The cell surface is less irregular in outline. The microvillous processes are not seen except as blunt cell projections (arrows). Within the cell the microfilaments (Mf) are clumped and disrupted. Higher up in the cells are a few tonofibrils (T) indicating the cell's epithelial origin.

X 29,900

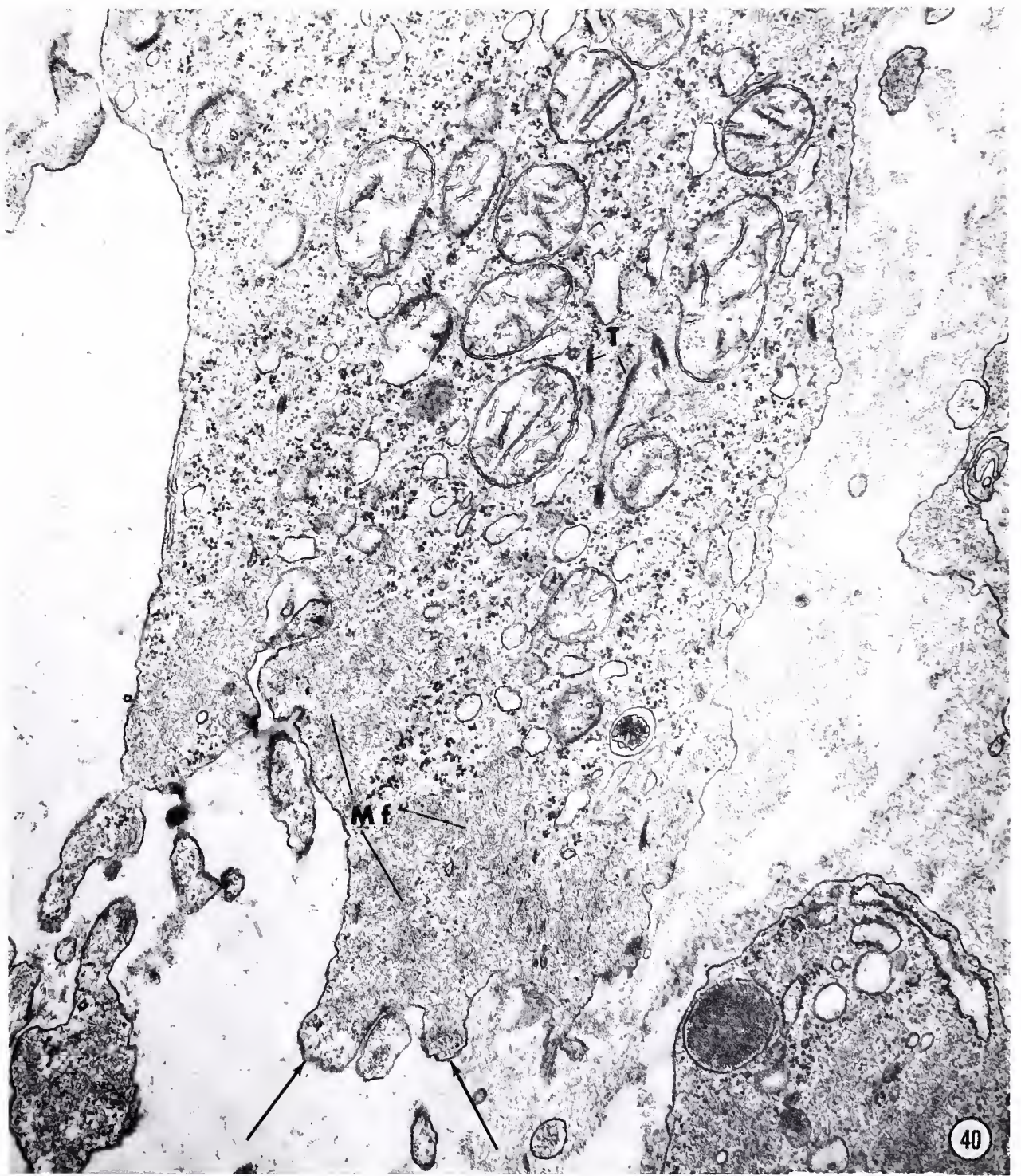


Figure 41. High magnification of Figure 40. The 40 Å microfilaments are clumped and disrupted. A vague suggestion of orientation, and polygonal web is still discernible. Dense clumps of amorphous material are seen (stars), and very small irregularly shaped electron dense particles (arrows) are seen scattered within the disrupted bundles of microfilaments. A small segment of a microtubule (Mt) appears in the upper part of the photograph.

X 78,700

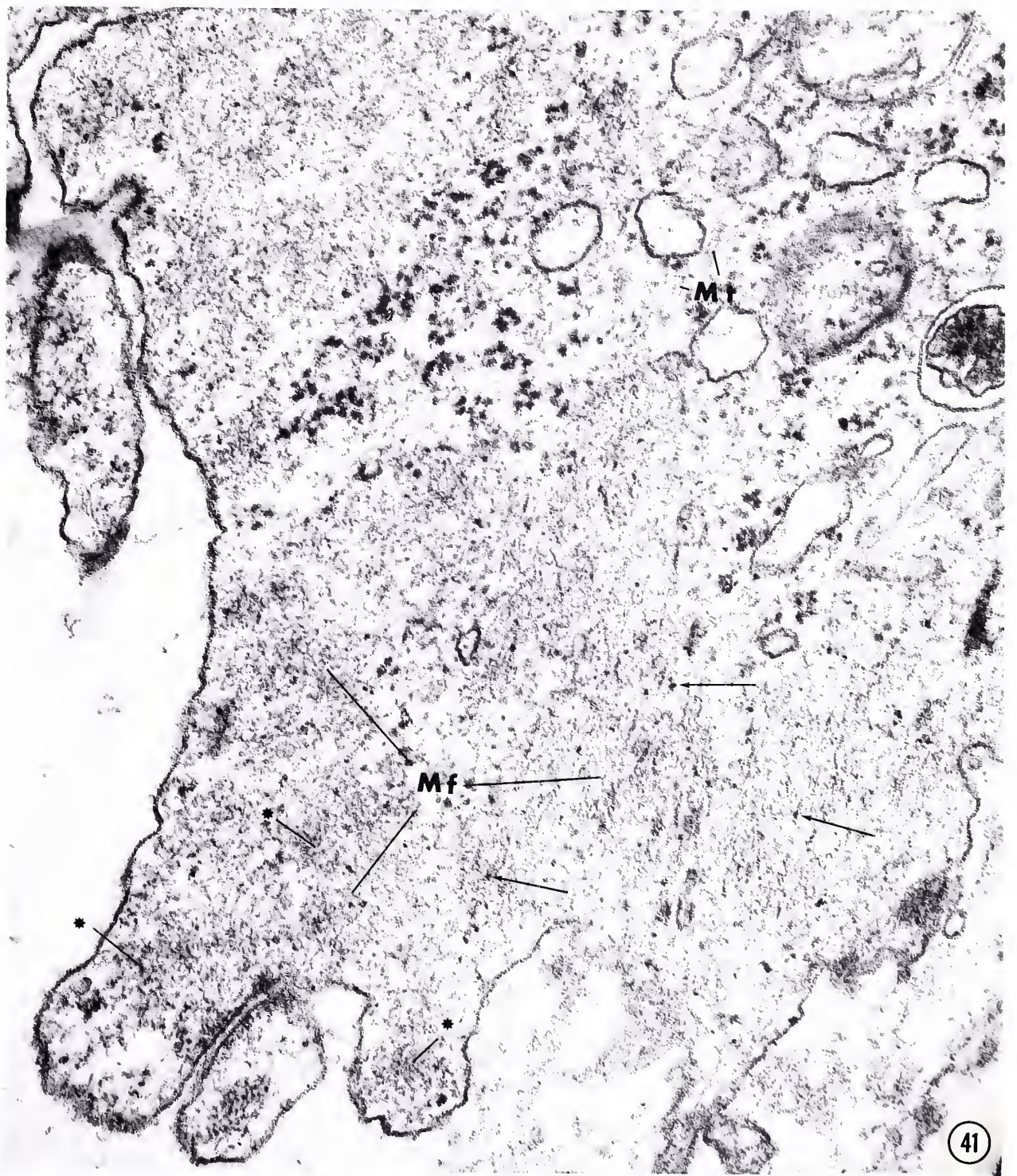


Figure 42. Control. 0.4% Dimethyl sulfoxide one hour in vitro. A part of a carcinoma cell is seen in the upper left. The 40 Å microfilaments (Mf) appear oriented parallel to the plasma membrane. On close examination, this consists of a regular array of polygons. This micrograph reveals the delicate polygonal web-like nature of these filaments particularly clearly. Some segments of microtubules (Mt) are oriented in the same direction as the microfilaments.

X 54,200

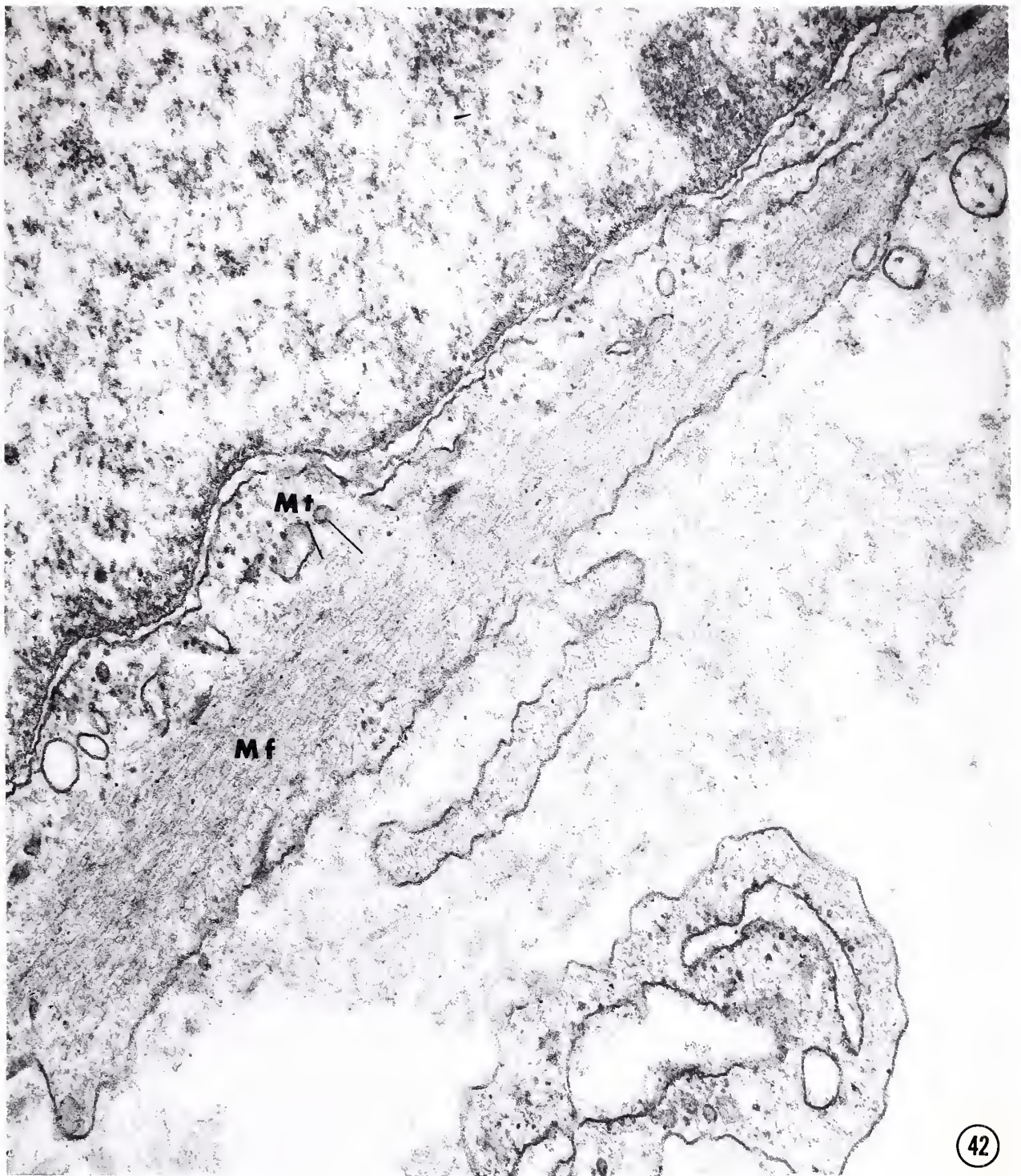


Figure 43. Cytochalasin B 100 $\mu\text{g/ml}$ in vitro, one hour.

This cell is similar to that seen in the control (Figure 41). The orientation of the microfilaments (Mf) parallel to the plasma membrane is only vaguely evident. The filaments are clumped (stars) and disrupted. The regular arrangement of polygons is less easily discernible. Again, small irregularly-shaped electron-dense particles (arrows) appear within the disrupted microfilament bundles.

X 70,600

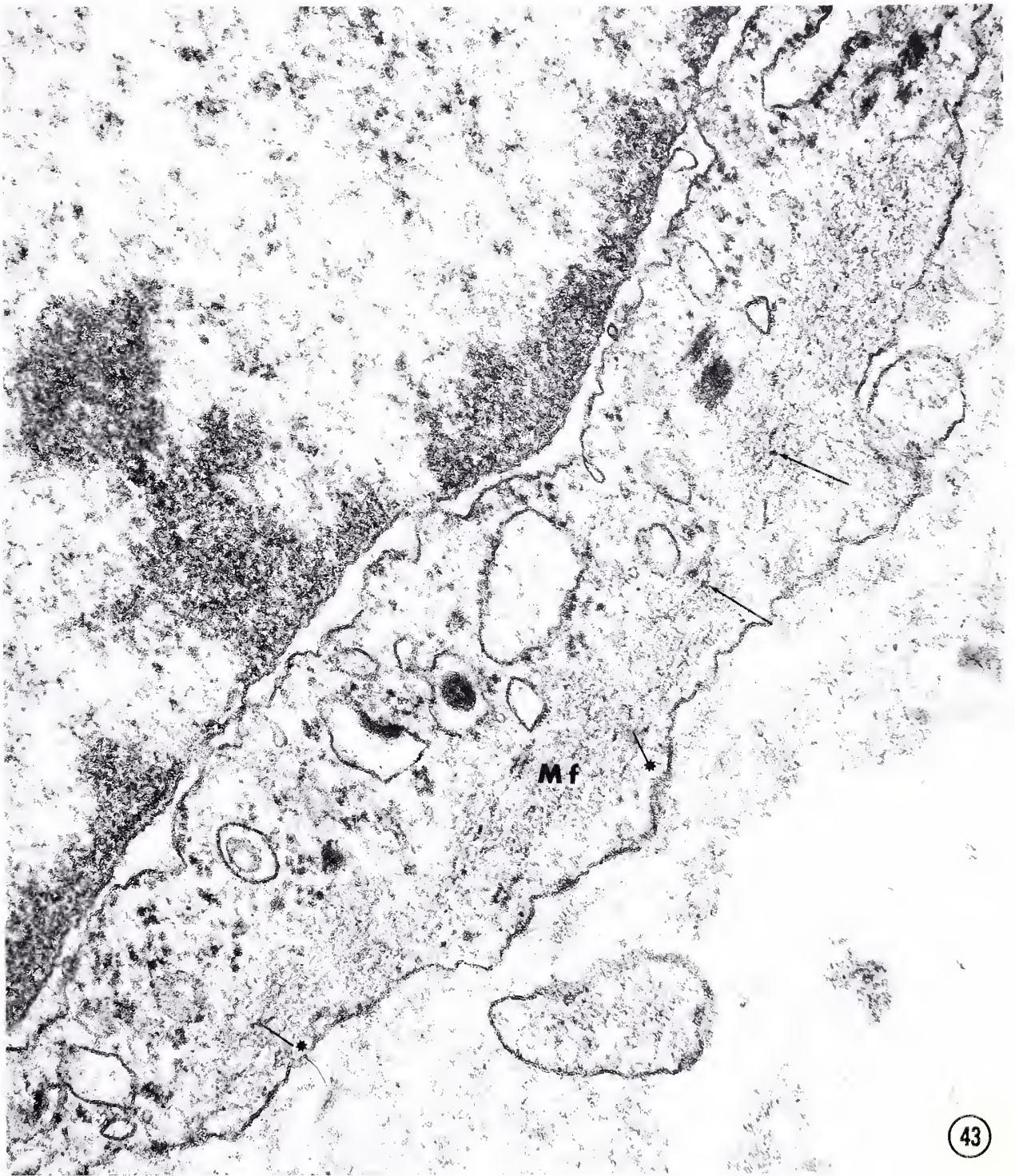


Figure 44. Cytochalasin B 100 $\mu\text{g/ml}$ in vitro one hour. Low magnification of a tongue of carcinoma cells extending into the connective tissue (CT). The microvilli (Mv) are blunted, and within the cells clumps of disrupted microfilaments (Mf) can be seen.

X 11,200

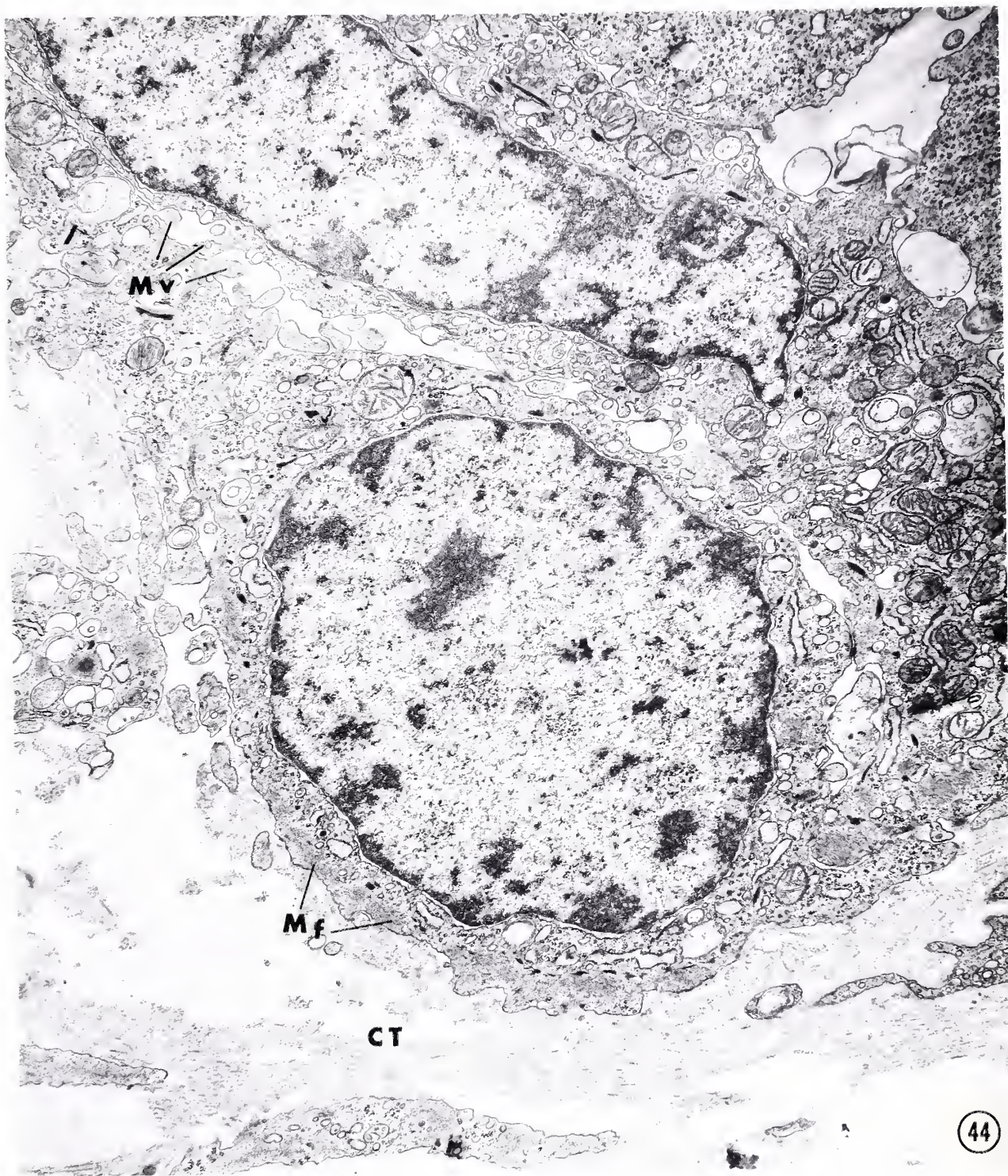


Figure 45. Control. 0.4% Dimethyl sulfoxide in vitro for one hour. High magnification of a microvillous process (Mv) containing 40 Å microfilaments (Mf). Again, a polygonal network can be discerned. In the upper cell process some of the 60 Å filaments (f) can be seen. These consist of straight filaments and do not form a polygonal network.

X 101,000

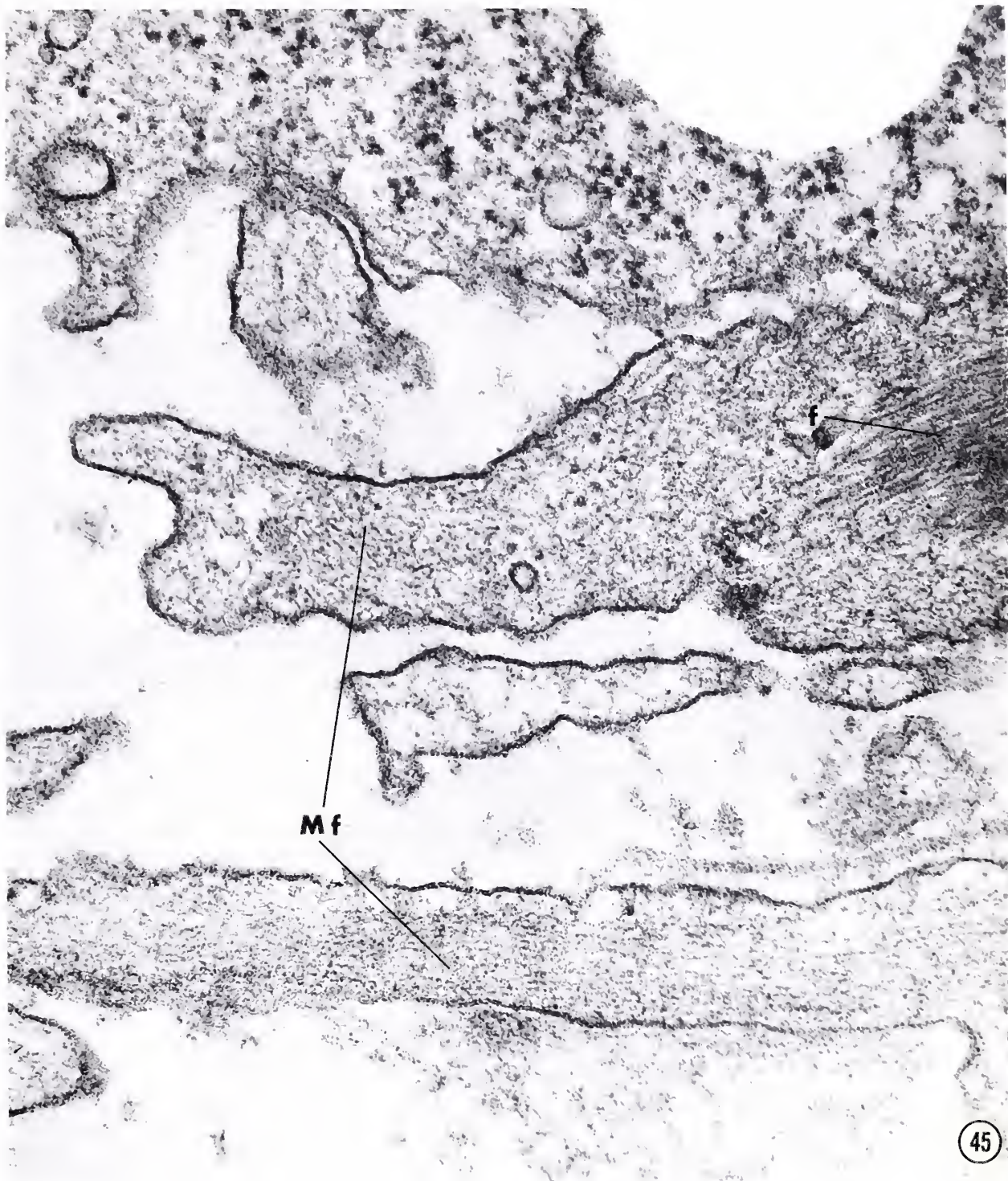
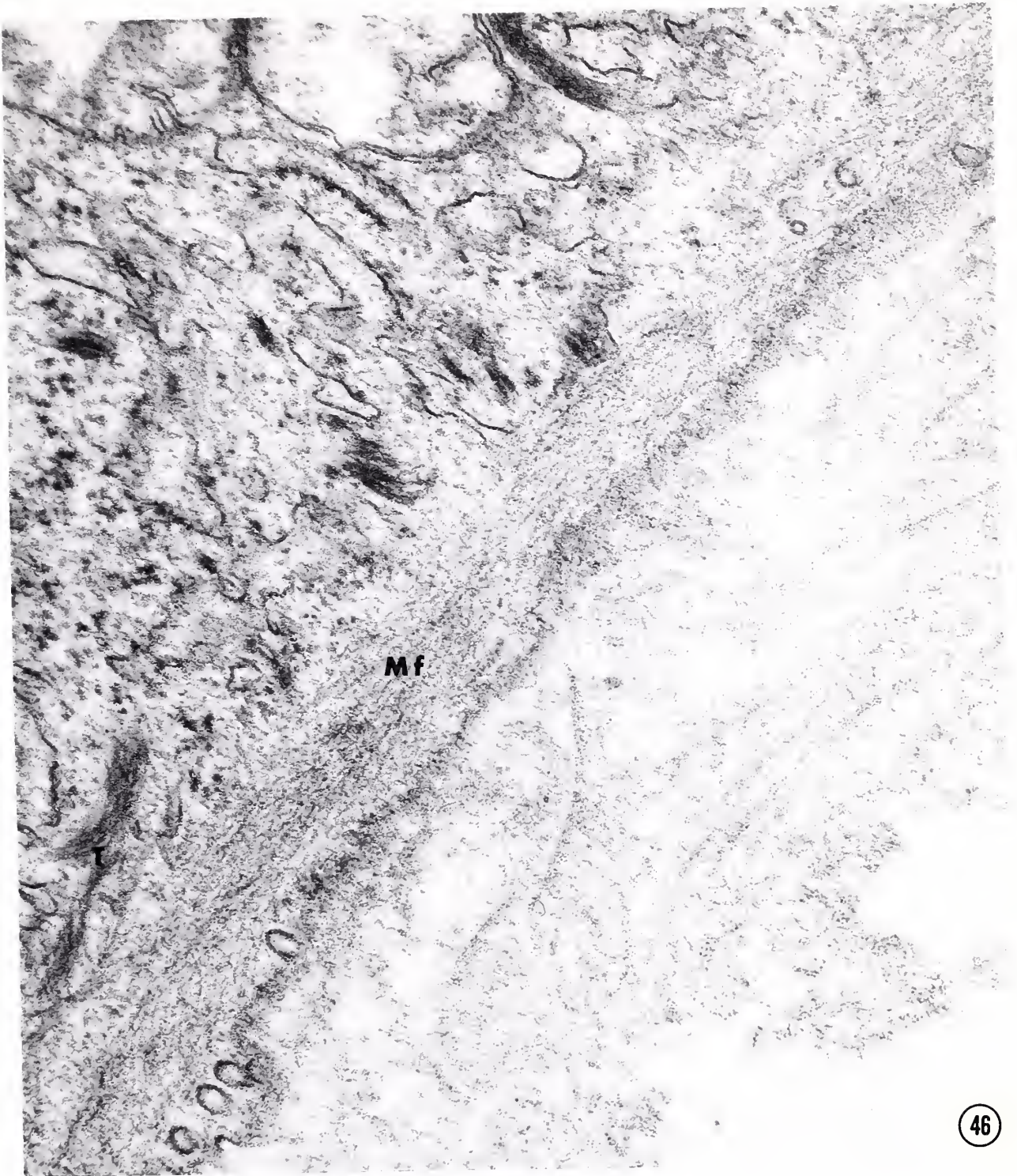


Figure 46. Control. 0.4% Dimethyl sulfoxide in vitro for one hour. High magnification of a section of a carcinoma cell cut obliquely to the surface of the plasma membrane. The cortical network of microfilaments (Mf) closely approaches the plasma membrane, seeming to merge with it. Other cyto-logic structures are excluded from the area occupied by these microfilaments. Short bundles of tonofibrils (T) are seen within the cell.

X 83,300



DISCUSSION

The results of these experiments indicate that the transformation of normal epidermis to invasive carcinoma involves the acquisition of cytological characteristics usually associated with active growth (20,88). During the development of a carcinoma, an increasing number of cells assume pleomorphic shape, undifferentiated cytological organization, and cortical microfilament networks. The latter have been noted previously in moving cells in culture (8,52,105) and migrating epidermis in healing wounds (64). The transformation to malignancy is also associated with increasing histological disorganization, widened intercellular spaces, a decrease in desmosome attachments, fragmentation and disappearance of basement lamina, and connective tissue destruction.

RIBOSOMAL PATTERNS

Early changes in cytological organization during carcinogenesis include a decrease in heterochromatin and the development of a prominent nucleolus, abundant free ribosomes, and endoplasmic reticulum. These changes have been described in other studies of transformation to malignancy (55,102,103). The appearance of prominent assymmetrically located nucleoli are indicative of the production of RNA necessary for the increased protein requirements of a rapidly growing tumor (19,27). At the

same time increased numbers of free cytoplasmic ribosomes can be seen in these cells. In the early stages of malignant transformation these occur singly, but in the more rapidly growing tumors they become associated into large clusters (polyribosomes or polysomes) (88). Free polysomes are often seen in rapidly growing cells and are indicative of protein production for use within the cell (20,88).

Membrane bound ribosomes (rough-surfaced endoplasmic reticulum) and Golgi complexes are associated with production of proteins to be used extracellularly (20,121). In the tumor cells there was a modest increase in rough-surfaced endoplasmic reticulum and vesicles and vacuoles of the Golgi complexes. The increase in this synthetic machinery may be associated with the appearance of the clumps of amorphous extracellular flocculent material (121).

In the more invasive tumors, areas of closely packed cells with the cytological organization described above alternate with areas of loosely associated more undifferentiated, pleomorphic shaped cells. The more undifferentiated cells differ from the more differentiated cells in that they contain fewer tonofibrils and their free ribosomes are less tightly associated into polysomes. This may indicate a decrease in protein synthesis compared to the more differentiated cells (89). The undifferentiated cells are of special interest because they also contain tapered pseudopods and long cortical networks of microfilaments. These cells are abundant in the invasive carcinomas and only rarely occur in the papillomas.

MICROFILAMENTS AND CYTOCHALASIN B

These experiments indicate that the appearance of microfilaments within the epidermal cells can be related to increasing malignancy. Normal untreated epidermal cells contain no microfilaments. With increasing malignant change almost all the epidermal cells can be found to contain a few short bundles of microfilaments near the plasma membrane or occupying the core of the numerous microvillous processes which appear at the cell surfaces. Within the invasive carcinomas, the pleomorphic shaped cells containing large cortical bands of microfilaments comprise a very significant part of the epidermal cell population.

Long cortical bands of microfilaments have been seen during cytokinesis (11,96,99,108,113), epithelial infolding in morphogenesis (14,31,98,104,119,127,128), in moving fibroblasts (8,52), glial cells (105), ascites tumor cells (45), and macrophages (9) in culture, and in the leading edge of growing axons (131,132). They have not been described previously in malignant cells in vivo. During wound healing, the migrating cells at the leading edge of the epidermis contain long cortical networks of microfilaments quite similar in appearance to those seen in this study (64). These cortical bands of microfilaments are of considerable importance because they may be related to the mechanism of cell movement. (See Introduction.) Some investigators have suggested that microfilaments are contractile and responsible for production of the force necessary for cell movement (61, 62,120).

A mechanism by which these filaments might interact to

produce contraction is not known. Some workers have found that the microfilaments of macrophages (9) as well as other cells (45,61,62,82) bind heavy meromyosin and thus may be similar to actin in both composition and function (9). Unequivocal evidence showing that microfilaments are contractile does not exist. There is a high degree of correlation between their presence and cellular processes such as motility which require a contractile mechanism. Thus, the occurrence of long cortical bands of microfilaments in many pleomorphic cells in the invasive carcinomas is indicative of a motile capacity of these cells.

The discovery of cytochalasin B, a fungal metabolite, has further implicated microfilaments in cell motility. Cytochalasin B in concentrations of 1 to 50 μg per ml. has been shown to prevent cell movement (30,47,105), cytokinesis (21,30,47,97,99), epidermal infolding (104,120,128), axón elongation (131,132), and endocytosis (9,118). In most of these processes cytochalasin either prevents the appearance of microfilaments or disrupts those already present at the same time that it exerts its effect on the particular cellular activity. These effects are reversible on removal of cytochalasin B and normal microfilaments reappear at the same time that the cell resumes normal activity.

The exact mechanism of action of cytochalasin B upon microfilaments is not known. It may act directly upon the microfilaments or cause disassembly of subunits (120). Other investigators believe that cytochalasin B may have a primary effect upon the cell surface (21,94). Recent experiments have suggested that cytochalasin B may selectively inhibit production

of mucopolysaccharides (94). The surface coats of cells contain glycoproteins and mucopolysaccharides (72,85) and appear to play a role in cell surface adhesiveness, especially the selective adhesiveness responsible for morphogenesis (39,40,80,91,114, 121). Cell surface adhesiveness is an important determinant of motility as seen in recent experiments (29,48,114). Other studies have revealed a high degree of correlation between the presence of a surface coat of mucopolysaccharide and areas of subsequent invagination of epithelium in salivary gland morphogenesis in organ culture. The cortex of these cells also contain bands of cytochalasin-sensitive microfilaments (17).

Cytochalasin B may alter surface properties or permeability by directly affecting the mucopolysaccharide coat (94) or surface membrane enzymes (92). These alterations in cell surfaces may secondarily result in morphological alterations in microfilaments and cessation of cell motility. Thus, the function of microfilaments in the processes of morphogenesis and cell motility has not necessarily been clarified by experiments with cytochalasin B. Even with this caution, the high degree of correlation between cytochalasin B-sensitive microfilaments and phenomena involving cell motility allows use of this chemical as a probe to determine the nature of the $40 \text{ \AA}$ microfilaments which become increasingly abundant in this study.

In the present study, the long cortical bands of microfilaments in the pleomorphic undifferentiated cells were disrupted by exposure to high doses of cytochalasin B. Since cytochalasin-sensitive filaments have been associated with morphogenesis and motility in other cells (17,47,97,99,104,105,

120,128,131,132) and cytochalasin has not been shown to affect other cellular filamentous processes such as tonofibrils or microtubules (104,120,128,131,132), it seems likely that these filaments in carcinoma cells are similar to those present in other motile cells. Malignant cells in general have the capacity to migrate or invade into adjacent tissues (123,124,125). Thus, these microfilaments are presumptive fine structural evidence of cellular movement during tumor invasion.

OTHER FILAMENTS

A. 60 Å Filaments

Within the more differentiated closely packed cells a band of peripheral filaments about 60 Å in diameter can often be seen parallel to and close to the plasma membrane. The bands are wider, straighter and lie deeper in the cytoplasm than the 40 Å microfilaments discussed above. This band of filaments is not sensitive to cytochalasin B and is morphologically similar to bands of filaments seen in fibroblasts (26,76). These fibroblast filaments have not been associated with motility. However, they appear in some studies to bind heavy meromyosin (61, 62). The relationship of these to the 40 Å microfilaments is not clear.

McNutt in a recent study (76) describes filamentous structures in normal 3T3, SV-40 transformed 3T3, and F1A revertent cell lines in culture. Two classes of filaments 70 Å and 100 Å are described. Contact inhibited 3T3 and F1A cells in a confluent monolayer contain many peripheral bands of 70 Å filaments, while the SV-3T3 cells which do not contact inhibit, and

non-confluent moving 3T3 and FlA cells contain markedly fewer 70 Å filaments. The 70 Å filaments very much resemble the bands of 60 Å filaments seen in the epithelial cells of this study in size, arrangement, and location. In addition, it is within the more differentiated closely packed cells of this study that these filaments appear. This is significant since these are the cells within the carcinoma which would most likely be under the influence of some contact inhibition mechanisms. McNutt may therefore be correct in assuming that these filaments are related to the process of contact inhibition. It is puzzling, however, that he did not report the presence of any filaments of smaller caliber in the moving non-confluent 3T3 or SV-3T3 cells that might correspond to the microfilaments seen in other moving cells. However, other investigators have described 40 to 60 Å filaments in moving fibroblasts (52).

A band of similar filaments has also been seen at the base of proximal tubule cells in the kidney in early stages of chemical carcinogenesis (55). They are also seen at the base of mammalian embryonic epidermal cells at some stages of development (23,63). The exact role of these filaments in all the cell systems described above is not apparent, but it is possible that they act as structural support when cells are no longer as well supported by the surrounding tissue.

B. Tonofibrils

Most of the changes in tonofibril patterns noted in this study have been described in detail in previous studies of experimental carcinogenesis (102). Of particular interest is that

the tonofibrils are reduced to a few very short spikes of material in the less differentiated, pleomorphic cells within the more malignant tumors. In normal epidermal cells and in the more differentiated cells of the papillomas the bands of tonofibers insert into desmosomes and hemidesmosomes, and probably provide strength and support to the epidermis (79,102). A breakdown of tonofibrils in the malignant cells to very short pieces indicates that the cells are not as firmly bound into sheets and are capable of more independent movement.

C. Microtubules

In the early phases of transformation there is an increase in the number of microtubules. With increasing malignancy, however, the number of microtubules decreases but they are still more numerous than in untreated epidermis.

When microtubules were first described, it was believed that they played an active role in ion transport (102). More recent experiments implicating them in changes in cell shape, elongation of axons, and orientation of directionality in moving cells have pointed toward a skeletal and orienting role for microtubules (22,50,84,116,131). The orientation of microtubules in cells seems often to presage the orientation of a cellular substructure or particular assymetrical cell shape.

Microtubules are seen in the flattening epidermal cells of the stratum germinativum (22), in growing nerve axons (131), and in motile cells in culture (50,116). In the early stages of carcinogenesis, the increase in microtubules is probably related to the rapid turnover of cells and rapid cornification of the

irritated epidermis. They may also be related to the need for structural support when widened intercellular spaces reduce supportive contacts between cells. In the motile cells of later stages of malignancy microtubules are probably playing a structural and orienting role, just as they do in motile cells in culture (50,116).

MITOCHONDRIA

The increased pleomorphism of mitochondria, the decrease in electron density of the matrix, and the appearance of an electron-dense round body in the matrix have been described previously (95,102,109). Investigators at first believed that the dense round body was a specific result of exposure to chemical carcinogens. Later studies have shown that treatment with irritative non-carcinogenic compounds could also result in the appearance of these inclusions (78,110).

MICROVILLOUS PROCESSES

In normal mouse skin the epidermal cells may be interdigitated but no microvillous processes are seen. Microvilli develop on the surface of the cells of the treated epidermis, even in the early stages of transformation. They are more prominent in cells where intercellular spaces are widened. Microvillous processes are a common feature of previous studies of malignant transformation (12,18,38,55,70,95,102,103,109), but are not a feature confined to malignant cells alone. Many normal cells in culture contain microvilli (35,44,111,112). The basal cells of human epidermis have some microvilli extending

into intercellular spaces (79). The periderm of embryonic epidermis contains many microvilli (23). The significance of microvilli in malignant epidermis is probably related to increased surface activity (44,112) and the need for an increased absorptive surface for the rapidly growing tumor cells.

In the present study some of the microvilli have a core of microfilaments which vary in their response to cytochalasin B. Microvilli of some cells seem to be affected by treatment with cytochalasin B and become blunted or disappear. Normal cells in tissue culture contain microvilli which are blunted by cytochalasin B treatment (51,99,118). This may be related to the 40 to 60 Å microfilaments seen in some microvilli (44,118).

PSEUDOPODIA

In the early phases of transformation and especially in the papillomas, an unusual type of basal cell extension can be seen protruding through the basement membrane into the connective tissue. These pseudopodia have narrow bases and ballooned-out tips. These pseudopodia contain very few microfilaments, and the cells from which they extend are predominantly more differentiated and closely packed. These pseudopodia have been seen in previous studies of chemical carcinogenesis and described in some detail (46,55,95,106,126). Some investigators have suggested that they represent epithelial invasion (55,106,109,126), and others have even suggested that the extensions pinch off and release lytic enzymes into the connective tissue, causing disruption of connective tissue elements (109).

There are several other circumstances unrelated to

carcinogenesis in which this type of cellular extension is seen. The chemical 3-hydroxyanisole has been shown to cause the appearance of this type of extension in vivo when the chemical is applied to the skin externally (100). It is not a carcinogen and its effects are reversible. No actual invasion of epidermis into underlying connective tissue occurs. Wessels has placed chick embryo epidermis over a millipore filter in culture and found that the cells over the holes in the filter form ballooned-out pseudopodial extensions at the base (63). Another circumstance in which this type of pseudopodia occurs is in wound healing (36,64). After spread of the epidermis over the disrupted connective tissue, ballooned out pseudopod extensions form at the base of the epidermis. Unlike the other pseudopods described above, these usually form temporary close contact (about 200 Å) with macrophages in the dermis. In addition this type of pseudopodia can be seen in the skin of the regenerating newt limb (93) and benign lesions of human skin (106).

In all these circumstances the pseudopodia do not result in invasion of underlying tissue by epidermis and do not appear to be a causative factor in any destruction of underlying tissue. During malignant transformation, the more differentiated cells form these extensions. In fact this type of activity is more common in the papillomas than in the malignant carcinomas (126). Finally, lysosomes usually associated with hydrolytic activity and lysis were not seen in these extensions. Thus, in this system they do not seem to play an important role in tumor cell invasion.

On the other hand, in the more invasive carcinomas, the

pleomorphic undifferentiated cells are commonly seen in areas of extensive connective tissue destruction. The pseudopodia of these cells are wide at the base, tapered toward the distal end, and contain many microfilaments. It seems likely that these pseudopodia are related to cell movement (53) and thus to tumor cell invasion.

DESMOSOMES AND OTHER SPECIALIZED JUNCTIONS

A decrease in the number of desmosome attachments between cells has been seen in previous studies of chemically induced malignant transformation as well as in natural carcinomas (12, 38, 46, 70, 77, 102, 103, 106, 126). In the early stages of transformation intercellular spaces are widened, but numerous desmosome attachments persist at the tips of drawn out spike-like cell processes. This type of configuration has also been noted in previous studies (102). This suggests that the cause for the early widening of intercellular spaces with carcinogen treatment is not secondary to a decrease in desmosomes. With increasing malignancy, however, there is a decrease in the number of desmosomes. They become very infrequent between the loosely packed undifferentiated cells of the invasive carcinomas.

Desmosomes are believed to play an important role in maintaining cell to cell adhesion (41, 42). A decrease in desmosomes and widening of intercellular spaces is probably the morphological basis for the decreased adhesiveness of tumor cells seen in early studies of neoplasia (32, 33, 34). Other studies have found a correlation between increasing invasiveness in vivo and the loss of cohesiveness of cells during the spread

of the initial outgrowth from a tumor explant in culture (13). Cells spreading out from an explant of an invasive carcinoma often break away from the main sheet of spreading cells and migrate independently on the culture surface. This is not seen with a spreading sheet of normal epithelial cells or cells from a benign epithelial tumor. Thus, the decrease in desmosomes seen in this study may imply a loss of adhesiveness between tumor cells allowing for more independent activity.

Both gap and tight junctions appeared to be decreased in the more malignant tumors. Some investigators have found specific decreases in the number of gap junctions (77) between epithelial cells of the cervix with increasing malignancy or a decrease in tight junctions between transformed non-contact inhibiting cells in culture (72). This has been felt to be of some importance since gap and tight junctions may be associated with decreased electrical resistance between cells, and therefore may be related to intercellular communication and contact inhibition (16,68,69). Other studies are less conclusive about the association between these junctions and contact inhibition (43).

EXTRACELLULAR MATERIAL

Clumps of amorphous electron-dense material are found in the widened intercellular spaces at all stages of transformation. Of special interest is the thin layer of flocculent material adherent to the surfaces of the pleomorphic undifferentiated cells of the carcinomas. Other investigators have found an increase in intercellular material in epithelial tumors (77). Some used ruthenium red as a stain for the surface coat of

cells, finding increases in cell surface coats to two or three times normal thickness after transformation with oncogenic viruses (72). Still others have found changes in the immunogenicity of cell surfaces after transformation (75). The importance of surface coats of glycoproteins or mucopolysaccharides in cell to cell adhesive specificity (80,91,92) and its possible importance in morphogenesis, contact inhibition, and cell motility (17,114,121) have been mentioned in the discussion of cytochalasin B. Thus, the appearance of this surface material may indicate changes in cell to cell adhesion and membrane surface activity in these undifferentiated pleomorphic cells.

BASEMENT LAMINA

Several studies have shown alterations, fragmentation and eventual disappearance of basement lamina in progressively more malignant epithelial lesions (12,46,55,70,95,106,109,126). Most of these investigators have suggested that the basement lamina is destroyed in some way by the rapidly advancing malignant epithelial cells. In the present study, also, there was a correlation between increasing malignancy and disappearance of basement lamina. However, even in the very invasive tumors, fragmented basement lamina may still occur under the more differentiated cells (12,70). The fragments tend to occur under and near hemidesmosomes. This correlation is of interest because basement lamina is a product of the epidermal cells (24,37,57), and production of new basement lamina by normal epidermis occurs first near hemidesmosomes (24,36). Basement lamina then spreads out over the remaining basal surface. Thus, the basement lamina in

malignant tumors appears in a location suggesting constant new formation. It is possible that the rapid growth, expansion and invasion of tumor into surrounding connective tissue results in many new areas of tumor-connective tissue junction devoid of basement lamina. The more differentiated cells respond by constant production of new basement lamina. In benign, non-invasive lesions, expansile growth alone may be responsible for small gaps in basement lamina (46,106).

Little or no basement lamina occurs in the vicinity of the pleomorphic, undifferentiated cells of the invasive tumors. These cells either are not producing basement lamina or are too actively invading the surrounding tissue for stable basement lamina to form beneath them. On the other hand, the adherent surface coats of these cells and the abundant extracellular material may represent synthesis of basement lamina components (eg. mucopolysaccharides) which have lost the capacity to form the ordered structure of the basement lamina.

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